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13. ABSTRACT ***(Maximum 200 Words)***

Our proposed research is focused on developing nitrone-based antioxidants as antidotes against chemical agents that induced excitatory neurotoxicity. We proposed to use kainic acid, an analog of the excitatory amino acid glutamate, to induce chronic neurological damage in adult rats. This model has been widely used as a model for studying human temporal lobe epilepsy. The delayed neuronal degeneration induced by kainic acid resembles CNS neuronal injury, repair, and plasticity. We have found that nitrone antioxidants, free radical trapping compounds, protected rats from kainic acid induced death and that co-treatment with the experimental antioxidant, phenyl-N-tert-butylnitrone (PBN) resulted in a diminution of NFkB, AP-1, and p38 activation, suppressed cytokine and apoptotic gene expression, inhibited neuronal apoptosis, and diminished seizure activity. These data suggest that pharmacological antagonism of multiple signal transduction pathways is achievable in the brain, and that inhibition of these processes may prevent a cascade of gene-inductive events leading to neuronal apoptosis. More recently, We have characterized other nitrone antioxidants, 2-hydroxy PBN, 3-hydroxy PBN, 4-hydroxy PBN, have similar effects. These results clarify the molecular basis for KA-induced seizure activity and may indicate a novel therapeutic strategy for certain chronic neurodegenerative disorders.

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Introduction:

The goal of our proposed research is to identify new strategies for inhibiting neuronal apoptosis, which occurs in neuronal trauma and degenerative diseases. We have begun to explore the molecular basis for apoptosis in kainic acid-induced neurodegeneration, a commonly used animal model for human temporal lobe epilepsy. Rats treated with kainic acid (KA) suffer recurrent convulsive seizures and apoptotic neuron loss in the CA1 and CA3 regions of the hippocampus. We hypothesized that KA chronically stimulates signal transduction pathways linked to apoptotic gene induction within sensitive populations of hippocampal neurons. In support of this hypothesis, we studied several distinct signal transduction pathways in the hippocampus following systemic exposure of KA. In particular, immunochemical studies and electromobility gel shift assays (EMSAs) demonstrate activation by KA of the NFκB (nuclear factor kappa B) system, the AP-1 (activator protein 1) system, and the p38 mitogen activated protein kinase (p38 MAPK) pathway. Most interestingly, treatment of the KA-exposed animals with the compound phenyl-N-tert-butylnitrone (PBN) inhibits KA-induced neuronal apoptosis, down-regulates apoptosis-associated gene expression, and prevents seizure activity and death.

Body:

This section of the report is associated with each task outlined in the approved Statement of Work

A. *Continue to elucidate the molecular mechanisms that underlie excitatory neurotoxin induced neurodegeneration. These will be assessed by using RNA protection assay for inflammatory cytokines and apoptosis-related genes (bcl 2, bax, caspase 1, 2, and 3), gel mobility shift assay for AP-1 and NFκB transcription factors, Northern and Western blot analyses for KA-induced mRNAs encoding Fos-related antigens, and c-Jun related transcription factors, and expression of inducible nitric oxide synthase (iNOS).*

To elucidate the molecular mechanisms that underlie excitatory neurotoxin induced neurodegeneration, we sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Fig. 5, Appendix 1), such as IL1α, IL1-β, IL-6 and TNF-α. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Fig. 6, Appendix 1). PBN treatment suppressed transcription of both inflammatory cytokine gene products and proapoptotic gene products; however, PBN treatment had a minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Figs. 5-6, Appendix 1). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcription, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Fig. 6, Appendix 1).

The immunochemical analysis of KA-treated rats was aimed at determining whether PBN could antagonize the AP-1 system *in vivo*. Immunocytochemical analysis was performed using well-characterized antibodies against the two AP-1 subunits, c-Fos and c-Jun. Within hours of KA treatment, c-Fos and c-Jun expression increased in hippocampal neurons, particularly within the CA1 and CA3 regions (Fig. 1). The c-Fos and c-Jun expression was maintained throughout the 4 day experiment (not illustrated), which is consistent with previously reported data (Bing et al., 1997). A single injection of PBN completely suppressed c-Jun expression in both CA regions and in the dentate gyrus (Fig. 1), but suppression of c-Fos expression only in the CA1 and CA3 regions, where most of the pathological changes manifested (Fig. 1). It may be significant to note that c-Jun expression can be induced rapidly in neurons during growth factor deprivation, but c-Fos expression seems to be restricted to those populations of neurons that actually commit to an apoptotic program (Estus et al. 1994).

B. Continue to elucidate the protective mechanisms of nitrone antioxidants in KA-induced neurodegeneration by characterizing the activation of p38 and JNK pathways as well as AP-1 and NFκB DNA binding activities.

The AP-1 pathway is but one of numerous signal transduction pathways which have been associated with cellular stress and linked to ligand-induced neurotoxicity. In particular, the p38 MAPK pathway has been repeatedly linked to neuronal apoptosis and, in some circumstances, may indirectly activate both the AP-1 and NFkB pathways (Schulze-Osthoff et al. 1997; Vanden Berghe 1998). The p38 mitogen-activated protein kinase pathway has been causally linked to neuronal apoptosis induced by growth factor withdrawal (Xia et al. 1995; Kummer et al. 1997). We therefore undertook an immunohistochemical analysis of p38 activation using an antibody specifically directed against the dual-phosphorylation motif which is only present on the active p38 kinase (Raingeaud et al., 1995). Within 4 hours of KA treatment, p38 activation was seen within the hippocampus in a pattern consistent with that of AP-1 activation (Fig. 3, Appendix 1). As in the case of AP-1, PBN suppressed p38 phospho-activation (Fig. 3 Appendix 1,). The p38 system remained activated somewhat above the level of controls at the four day timepoint, but this chronic activation was not as dramatic as in the AP-1 case (not shown).

The NFκB transcription factor is also ubiquitously activated by physiologic stress and may potentiate excitotoxic damage in striatal neurons (Qin et al. 1998). Alternatively, NFκB seems to serve a protective role in hippocampal neurons undergoing an oxidative insult (Mattson et al. 1997) and may actually play an anti-apoptotic role in TNFα-stimulated cells (Van Antwerp et al. 1996; Wang et al. 1998). NFκB is part of a signal transduction cascade which has traditionally been thought of as distinct from the Jnk and p38 cascade modules, though correlated activation of the three pathways is often noted in cell culture experiments. We therefore sought to determine whether NFκB was activated by KA in a PBN-sensitive manner. As shown in Fig. 4, NFκB-p65 immunoreactivity in the hippocampus increased dramatically within hours of KA treatment, and this effect was suppressed by PBN. The immunochemical data corroborated by EMSA analysis, showed a dramatic increase of NFκB binding activity

in hippocampal nuclei of KA treated rats, which was partially mitigated by PBN cotreatment (Fig. 4, Appendix 1).

C. *Test several other nitrone antioxidants for the protective action against kainic acid induced neuro-degeneration. In previous studies, we have tested several PBN derivatives for anti-inflammatory activities using a macrophage system. Based on the results of this cellular screening we selected four PBN type nitrones having a substituted phenyl group which showed the most effectiveness in neuroprotective actions. These are 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN), 2-sulfo- PBN (2-SPBN), and salicyl t-butylnitrone (SALBN).*

We have studied other nitrone antioxidants for the protective action against kainic acid. Those were 2-hydroxy PBN (2-OHPBN), 3-hydroxy PBN (3-OHPBN), 4-hydroxy PBN (4-OHPBN), 2-sulfo-PBN (2-SPBN). However, all of above-mentioned compounds had less effect than PBN in vivo. This may be partially due to the brain blood barrier that these reagents have to pass to have an effect on the hippocampal neurons. We will try to use our established neuron/glial mixed culture to test these reagents since they have been tested in our microglial culture system and showed anti-inflammatory effects.

D. *Additional findings:*

In our searching for transcription factor in KA induced neurodegeneration, we have collaborated with Dr. Feng to successfully cloned a fos-related antigen. This is a transcriptional factor that also related to guanosine triphosphate regulation (Appendix 2, P.I. as a corresponding author). With a full length cDNA (Fig. 1, Appendix 2) and Gel retardation analysis (Fig. 4, Appendix 2), we were able to reveal that this gene may function as a GTP regulation related factor.

Key Research Accomplishment:

- **Established kainic acid induced neuronal damage in adult rats as a model to study excitatory amino acid-induced neurodegenerative diseases by Terminal deoxyuridine nick-end labeling (TUNEL) for apoptotic cell death, Nissl staining and immunohistochemical assays.**
- **Demonstrated that nitrone antioxidant, PBN, inhibits KA_induced neuronal apoptosis, down regulates apoptosis-associate gene expression, and moreover, prevents seizure activity and death.**
- **Elucidated the molecular mechanisms underlying the nitrone antioxidants' protective functions against KA-induced neurodegeneration with signal transduction pathways by studying the activation of NFkB, p38, and AP-1.**
- **Tested several other PBN related antioxidants in kainic acid induced neurodegeneration.**
- **Cloned a new gene that related to GTP regulation**

Reportable Outcomes:

Reprint:

Abstract and Presentation:

Animal Model: We have successfully used KA-induced neurodegeneration as a animal model for delayed neuronal cell death that occurred in many neurodegeneration diseases such and Alzheimer's and Parkinson's diseases.

Cloning of a new gene: Cloned MP 13 gene from rat hippocampus after kainic acid treatment.

Conclusion:

The findings of the present study extend upon previous observations concerning the broad-spectrum neuroprotective action of nitrone compounds, and provide a novel context for discussing the pathology of excitotoxicity. PBN and related nitrones have been shown to suppress striatal excitotoxic lesions induced by KA. The present data

suggest that suppression of apoptosis by PBN in the KA model and possibly other models of neurodegeneration is likely due to mitigation of proinflammatory or proapoptotic gene expression under the control of the AP-1, NFkB, and p38 MAPK pathways. While the ultimate cellular target(s) for PBN action remain unclear, the present data suggest that the broad-spectrum neuroprotective action of the nitrone class of compounds might be due, in part, to antagonism of crucial oxidation-sensitive signal transduction elements linked to the initiation of apoptotic programs.

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Appendices: Reprints, abstracts, CV.

1. Floyd, R.A., Hensley, K., **Bing, G. (2000)** Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics. *J. Neural Transm.* 60:387-414.

2. Feng, Z., Qi, Q., Wilson, B., McMillan, M., Kim K.H., Hong, J. **Bing, G. (2000)** Cloning and expression of MP 13, an antigen immunoreactive with antibody against FOS-related antigen, from rat hippocampus after systemic kainic acid treatment. . *Neurosci Lett.* 296:129-132.

3. Fig. 1

4. Abstracts.

5. C.V. for Guoying Bing

375.3

PHENIDONE PREVENTS KAINATE-INDUCED NEUROTOXICITY VIA ANTIOXIDANT MECHANISMS. H.C. Kim[1], W.K. Jhoo[1], G.Y. Bing[2], E.J. Shin[1], M.B. Wie[3], W.K. Kim[4*], K.H. Ko[5]. [1]*Neurotoxicol. Prog., Dept. Pharmacy, Coll. Pharmacy, Kangwon National Univ., Korea Institute of Drug Abuse, Chunchon, North Korea,* [2]*Free Radic. Biol.& Aging Res. Prog., OMRF, Oklahoma City, OK, USA,* [3]*Dept. Vet. Med. Inst. Life Sci., Cheju National Univ., Cheju, North Korea,* [4]*Dept. Pharmacol., Coll. Med., Ewha Med. Res. CTR., Ewha Womans Univ., Seoul, North Korea,* [5]*Dept. Pharmacy, Coll. Pharmacy, Seoul National Univ., Seoul, North Korea*

In order to extend our understanding of the pharmacological intervention of phenidone, we evaluated antioxidant activity of this compound *in vivo* in the present study. In order to better understand the significance of a blockade of both cyclooxygenase and lipoxygenase pathways, we studied the effects of aspirin {ASP; a non-selective inhibitor of cyclooxygenase), NS-398 (a selective inhibitor of cyclooxygenase-2), esculetin (a inhibitor of lipoxygenase) and phenidone (co-inhibitor) on lipid peroxidation, protein oxidation, and glutathione (GSH) status in the rat hippocampus after KA administration. ASP, NS-398, esculetin or phenidone was administered orally five times every 12 h before the injection of KA. The KA-induced toxic behavioral signs, oxidative stress, impairment of GSH status, and the loss of hippocampal neurons were significantly attenuated by the phenidone in a dose-dependent manner. However, ASP, NS-398 and esculetin failed to protect against the neurotoxicities induced by KA. Therefore, the results suggest that blockade of both cyclooxygenase and lipoxygenase pathways are responsible for KA-induced neuroexcitotoxicity via antioxidant machanisms. *Supported by: BK 21 project, and a grant (#HMP-98-N-2-0013) of the Good Health Research and Development Project (1998) of Ministry of Health and Welfare, Republic of Korea.*

379.14

PROLONGED EXPOSURE TO β-AMYLOID PROTEIN ENHANCES 4-HYDROXY-2-NONENAL MODIFIED PROTEINS IN THE RAT BRAIN. W.K. Jhoo[1], H.C. Kim[1], K. Yamada[2], D.H. Im[1], E.J. Shin[1], S.J. Park[1], G.Y. Bing[3], K.J. Jang[1*], T. Nabeshima[2]. [1]*Neurotoxicol. Prog., Dept. Pharmacy, College of Pharmacy, Kangwon National University, Korea Institute of Drug Abuse, Chunchon, Korea,* [2]*Dept. Neuropsychopharmacology and Hospital Pharmacy, Nagoya University School of Medicine, Nagoya, Japan,* [3]*Free Radic. Biol. & Aging Res. Prog., Oklahoma Medical Research Foundation, Oklahoma City, OK, United States of America*

We report increased modification of proteins by 4-hydroxy-2-nonenal(HNE), a product of membrane lipid peroxidation in the rat brain following continuous infusion of β-amyloid protein-(1-42) into the cerebral ventricle. A mild HNE immunoreactivity was induced in the pyramidal neuron of the cortex, Ammon's horn and dentate granule cells of hippocampus and thalamic area in the vehicle or β-amyloid protein-(40-1)-infused brain. However, the induction of HNE immunoreactivity was more pronounced in the Ammon's horn of hippocampus and thalamic area following prolonged infusion of β-amyloid protein-(1-42). HNE-immunoreactive astrocytes were proliferated mainly in the stratum radiatum of the CA1 sector without significant neuronal degenerations. Consistently, the immunoreaction with 8-hydroxyguanosine (a marker of oxidative damage to DNA and RNA) was enhanced in these brain regions after chronic exposure to β-amyloid-(1-42). Therefore, our findings suggest that β-amyloid protein- mediated oxidative stress leads to the productions of HNE and 8-hydroxyguanosine in the rat brain.

381.4

THE EFFECT OF INTERLEUKIN-10 ON LIPOPOLYSACCHARIDE-INDUCED NEURODEGENERATION IN SUBSTANTIA NIGRA DOPAMINERGIC NEURON. T. Arimoto, X. Lu, C.A. Stewart*, G.Y. Bing. *Free Radical Biology and Aging, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA*

Injection of the bacteria endotoxin lipopolysaccharide (LPS) in substantia nira (SN) resulted in the activation of microglia and the significant loss of dopaminergic neurons. The apparently, inflammatory processes including immune responses play an important role in the development and progression of neurodegeneration. The present study is to examine whether LPS-induced neurodegeneration could be attenuated by interleukin (IL)-10, a global inhibitor of cytokine synthesis. Single intranigral injection of IL-10 (44 ng) or osmotic pump infusion (60 ng/day) for 14 days in right SN before LPS (5 μg) increased tyrosine hydroxylase-immunoreactivity in the SN. Ribonucrease protection assay revealed that LPS treatment in right SN significantly induced mRNA for IL-1β, IL-6, and TNF-α at 24 hrs after the injection, as compared the left side. These cytokine expressions were significantly inhibited by IL-10 injection before LPS treatment. These results indicate that IL-10 could protect against LPS-induced neurodegeneration in SN through the down-regulation of inflammatory cytokine expression. (This study was supported by USAMRMC 98228027 and R01 NS39345 Granted to GB)

481.19

ENHANCED EXPRESSION OF MICROSOMAL EPOXIDE HYDROLASE IN RAT ASTROCYTES BY LIPOPOLYSACCHARIDE AND INFLAMMATORY CYTOKINES: A TISSUE-SPECIFIC REGULATION. A.Y. Sun[1], N. Zheng[1], M. Zhu[1], M. West[1], H.C. Kim[2], G.Y. Bing[1*]. [1]*Free Raddical Biology and Aging, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA,* [2]*College of Pharmacy, Chunchon, South Korea*

Microsomal epoxide hydrolase (mEH) is a critical biotransformation enzyme that plays a central role in the metabolism of xenobiotics. In present study, mEH levels in cultured cortical astrocytes were detected by Western blotting, and the effect of lipopolysaccharide (LPS) and cytokines on mEH expression were examined. Quiescent astrocytes express basic level of mEH, with apparent molecular weight 50 kDa. but their levels varied with the growth status. Incubation of astrocytes with LPS and cytokines caused a dose-dependent increase in mEH levels. LPS (100 μg/kg), IL-1 β (10 ng/ml), or TNF-α (0.5 μg /ml) induced a 3-5 fold increase of mEH. A time-course analysis revealed that induction of mEH proteins by LPS was evident after 24 h treatment, and reached a maximum after 72 h. IL-1 β induced a response of mEH expression much earlier than TNF-α, with the maximal responses observed after 24 h and 72 h, respectively. By contrast, interferon-γ (1 μg/ml) only showed a small effect on mEH expression. In addition, combinations of IL-1 β and TNF- αelicited an induction of mEH expression quite similar to the temporal parttern of mEH expression induced by LPS. These results provide the first demonstration that pro-inflammatory factors differentially regulate the xenobiotics biotransformation enzymes, with an induction in CNS astrocytes in contrast to depression in liver. The potential functions of mEH up-regulation in neurodegenerative diseases therefore warrant a further investigation. (This study was supported by USAMRMC 98228027 and R01 NS39345 Granted to GB)

390.6

LONG-TERM ELEVATION OF GLUTATHIONE S-TRANSFERASE YC SUBUNIT IN RAT HIPPOCAMPUS AFTER KAINATE INJECTION. L. Jin*, N.Y. Zheng, M. Zhu, R. Nael, L.L. Zhao, G.Y. Bing. *Free Radical Biology and Aging, Oklahoma Med Res Fndn, Oklahoma City, OK, USA*

Our previous work has shown that differential expression of the genes after kainic acid (KA) injection may underlie the molecular mechanisms for spontaneously convulsive seizure activities. Using the suppression subtractive hybridization (SSH) and PCR-select differential screening methods (Clontech, Palo Alto, CA). We have found more than 120 genes that have been differentially regulated by KA injection. Glutathione S-transferase Yc subunit (GSTYc) was found to be one of the genes that shown long-term elevation. Northern Blot analysis of the total RNA isolated from rat hippocampus 6 h, 1 day, 1 week , 2 weeks, and 3 months after KA (n=3 for each time points) showed that GSTYc was initially down regulated at 6 h and 1 day, but dramatically increased 1 week after KA injection and persisted at higher level for at least three months. In situ hybridization for GSTYc revealed a marked increase of GSTYc in both neurons and glial cells at 2 weeks and 3 months after KA injection. The present study indicated that free radical damage may involve in KA-induced recurrent convulsive seizure activities. These results suggests that GSTYc subunit may play an important role in long-term pathophysiological changes in rat model of human temporal lobe epilepsy. (This study was supported by the OCAST HR98-004, USAMRMC 98228027, and R01 NS39345 Granted to GB)

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Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics

R. A. Floyd[1,2], **K. Hensley**[2], and **G. Bing**[2]

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[2]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, U.S.A.

Summary. A brief review is presented on observations leading to the current notions regarding neuro-inflammatory processes. The greatest focus is on Alzheimer's disease (AD) since this is where the most convincing data has been obtained. A brief summary of observations on the neuroprotective action of α-phenyl-*tert*-butyl-nitrone (PBN) as well as results of research designed to understand its mechanism of action is presented. We hypothesize that the mechanism of action of PBN involves inhibition of signal transduction processes, which are involved in the upregulation of genes mediated by pro-inflammatory cytokines and H_2O_2 that cause formation of toxic gene products. Results from recent experiments on Kainic acid (KA) mediated brain damage are provided to suggest the validity of the in vivo action of PBN to inhibit neuro-inflammatory processes. The accumulating scientific facts are helping to provide concepts that may become the basis for novel therapeutic approaches to the treatment of several neurodegenerative diseases.

Introduction

Our attempts to explain the serendipitous observations made on the neuroprotective action of α-phenyl-*tert*-butyl-nitrone (PBN) several years ago provided a challenge, which lead us to postulate the occurrence of neuro-inflammatory processes in the stroked and the aging brain to help explain the results. Surprising observations made earlier by other investigators also forced them to conclude that enhanced neuro-inflammatory processes occur in the Alzheimer's Disease (AD) brain. Observations we made recently, combined with the early seminal findings and the many others made since, overwhelmingly support the notion that neuro-inflammatory processes occur in the AD brain. Results obtained in our attempts to explain the mechanistic basis of the neuroprotective action of PBN provide strong support for the notion that this compound acts, not by trapping free radicals in a mass-action

transduction processes in the classic injury cascade and also activate complement, which results in an amplified β-amyloid AD cascade". Their work shows different cell types collaborate and amplify the β-amyloid triggering events. Mediators generated by microglia (IL1, TNFα) activate astrocytes to produce other factors (IL6, etc.) that further activate nearby cells (Cotman et al., 1996). Thus β-amyloid plaques become "sparking centers" for what turns out to be "localized smoldering neuro-inflammatory processes" (Floyd, 1999a). Very recent research pertinent to the molecular events triggering the localized neuro-inflammatory processes have demonstrated that β-amyloid activation of microglia involves the interaction of CD40 receptor and the CD40 ligand (Tan et al., 1999).

Enhanced reactive oxygen species and oxidative damage are consequences of neuro-inflammatory processes

Enhanced reactive oxygen species (ROS) and the resulting oxidative damage is a characteristic feature of the AD brain (Markesbery, 1997; Smith et al., 1991; Smith et al., 1996). This is probably the result of several neuro-inflammatory events where ROS are known to be produced in excessive amounts. Activated microglia produce high levels of superoxide (Colton and Gilbert, 1987). β-amyloid activates microglia, monocytes and neutrophils to form superoxide via the NADPH oxidase pathway (Bianca et al., 1999). The amount of superoxide formed, measured as H_2O_2, was on the order of 1 nmole H_2O_2 per 3×10^5 cells in 30 minutes when stimulated with 10 μM β-amyloid peptide. β-Amyloid peptides per se also degrade to form ROS (Hensley et al., 1994), specifically H_2O_2, through transition metal ion reductive processes (Huang et al., 1999). Amyloid precursor protein per se regulates copper toxicity to neurons (White et al., 1999). H_2O_2 production by β-amyloid peptides per se or by the peptides interacting with microglia may be very important in triggering glia activation processes. We have shown that H_2O_2 itself activates cultured rat astrocytes in a manner very much like IL-1β (Robinson et al., 1999a). Clearly then H_2O_2 itself becomes a neuro-inflammatory propagating agent.

Enhanced protein oxidation associated with AD and in aging brain

Enhanced ROS formation would be expected a priori to lead to enhanced protein oxidation as well as enhanced lipid peroxidation. Significantly higher levels of protein oxidation have been noted in the AD brain versus the age-matched control brain (Smith et al., 1991). It was noted that specific brain regions had higher amounts of oxidized proteins. In general, those regions most affected by AD had higher levels of protein oxidation. It was also noted that protein oxidation increased logarithmically with age in normal, i.e. non-AD subjects. This seems to be a characteristic feature of brain aging. Increases in oxidized protein in brain with age have been noted in many

experimental models (Stadtman, 1992), including mice (Dubey et al., 1996; Forster et al., 1996), rats (Dubey et al., 1995), and gerbils (Dubey et al., 1995; Carney et al., 1991).

The increased levels of oxidized protein in brain with age could be due to a decrease in the rate of breakdown of oxidized protein by proteases. The research of Agarwal and Sohal (1994) addressed this possibility. Their results show that brain alkaline protease activity, the protease fraction shown to be responsible for the breakdown of oxidized protein, see references (Oliver et al., 1984; Mason and Rivett, 1994; Rivett, 1985; Rivett, 1989), does not decrease with age (Agarwal and Sohal, 1994). From this data they concluded decreases in alkaline protease activity could not explain the age-related increase in oxidized protein in brain. The point of this discussion is an attempt to rationalize the data obtained on the neuroprotective activity of PBN in different models and its affect on brain oxidized protein in rat and gerbil brain in relation to its proposed action of suppressing signal transduction processes. The reason why PBN suppresses the amount of oxidized protein in the aged gerbil brain (Carney et al., 1991; Floyd and Carney, 1996) may be because it suppresses the signal transduction processes leading to increased ROS generation caused by the inherent (unknown) activation processes that occur with age. In contrast to rats and gerbils it was noted, in the only study published, that the administration of PBN to older mice did not cause a significant reduction in oxidized protein in cerebral cortex (Dubey et al., 1995). A careful review of that work showed that there was a trend toward PBN-mediated reduction in oxidized protein, but it was not large enough to be significant. This may be because cerebral cortex is a brain region in mouse that does not change greatly in oxidized protein with age as other brain regions (Dubey et al., 1996; Foster et al., 1996) and possibly because the mice in the study were significantly younger (23 months) than the other studies where older mice were used. Additionally, the mice were administered PBN as bolus injections (32 mg/kg). Administration of it in drinking water, a regiment that has been shown to prolong life span in mice (Saito et al., 1998), may have been more effective.

Neuro-inflammatory processes in the aging brain

There are only a few studies in experimental animals directed toward the examination of the normal aging brain from the perspective of evaluating if neuro-inflammatory type processes occur. However, these studies do provide strong evidence to support the notion that neuro-inflammation type processes are present and do increase with age. Recent detailed studies in this area have come from Finch's lab (Rozovsky et al., 1998; Morgan et al., 1999) and from Morgan's lab (Gordon et al., 1997). The older literature was referenced by Finch and Morgan (1990). The results are consistent in showing that aging in brain is associated with an increased expression of glial fibrillary acidic protein (GFAP); and that increased GFAP expression is a marker of astrocyte activa-

tion and is a response to CNS injury. Gordon et al. (1997) showed that injury, induced by several means, including 6-hydroxy-dopamine injection or a needle stab wound, to the old brain, caused a more exaggerated astrocyte response, which persisted much longer than the same injury did in a young brain. So the old brain responded more to an injury and the response to that injury persisted for much longer. These studies reinforce the results of our work in gerbils where we noted that a stroke insult was much more serious to older animals (Carney et al., 1991; Floyd, 1990). In a careful study where microglia and astrocytes were collected from 3-, 6-, 12- and 24-month rat brains, Rozorsky et al. (1998) demonstrated that both microglia and astrocytes taken from old brains had more proliferative capacity and expressed more GFAP than those taken from young brains. TGF-β_1, which normally down-regulates inflammatory processes was less capable of suppressing proliferation of astrocytes and microglia taken from older brains when compared to younger brains (Rozovsky et al., 1998). Similarly TGF-β_1 was less capable of suppressing LPS-induced nitrate formation in the cultured microglia from older brains than the microglia from younger brains. Their data was interpreted as supporting the "hypothesis that aging promotes a proliferative microenvironment in the brain".

Excess nitric oxide and peroxynitrite reaction products in AD brain

Products formed by the reaction of nitric oxide (NO) and peroxynitrite, (formed by the reaction of NO with superoxide), with cellular components were shown to be enriched in the affected regions of the AD brain (Smith et al., 1997; Hensley et al., 1998). This is also clear evidence of the involvement of neuro-inflammatory processes in the AD brain. It is known that pro-inflammatory cytokines as well as β-amyloid stimulates the production of NO in astrocytes (Akama et al., 1998). β-Amyloid enhanced NO production by astrocytes involves NFκB-mediated mechanisms (Akama et al., 1998). Enhanced NO production most likely occurs because of the induction of inducible nitric oxide synthase (iNOS) which mediates the formation of large amounts of NO. Utilizing three different antibodies to 3-nitro-tyrosine, (a product formed by the reaction of peroxynitrite with protein tyrosines), Smith et al. (1997) demonstrated significant 3-nitro-tyrosine staining in affected regions of AD brain but none in comparable age-matched control brain regions. Using novel HPLC-electrochemical detection methods to quantify the 3-nitro-tyrosine content of protein digest, we demonstrated that the content of this nitrative adduct is increased 3 to 7-fold in affected brain regions of AD subjects when compared to age-matched control brain regions (Hensley et al., 1998). In addition to 3-nitro-tyrosine, we simultaneously measured the dityrosine content of the protein digest and noted that this adduct followed in a somewhat general pattern to that observed for 3-nitro-tyrosine content (Hensley et al., 1998). Dityrosine adducts are formed by the bimolecular addition of tyrosyl free radicals.

Nitric oxide and peroxynitrite is more toxic to neurons

The clear demonstration of enhanced NO formation in affected regions of AD brain evokes a possible mechanistic basis for the mediation of neuron death or dysfunction. It has been shown that NO (and its reaction products) is more toxic to neurons than to the glia which produces it in copious quantities (Dawson et al., 1993; Dawson and Dawson, 1996). Study of the neurotoxic potency of NO and its reaction products have shown that its reaction with superoxide to form peroxynitrite is a key event in its neurotoxicity (Lipton et al., 1993). The exact molecular events involved in the neurotoxicity of nitric oxide and reaction products are not known.

Enhanced signal transduction processes near β-amyloid plaques

The involvement of neuro-inflammatory processes surrounding β-amyloid plaques is expected to cause enhanced intracellular signaling (signal transduction processes) in cells surrounding the plaques (Cotman et al., 1996). Enhanced signal transduction processes are expected because, as noted previously, β-amyloid has been shown to activate microglia via the CD40/CD40L complex (Tan et al., 1999) and to mediate formation of H_2O_2 by microglia (Colton and Gilbert, 1987) as well as to produce H_2O_2 itself (Huang et al., 1999). H_2O_2 has been shown to mediate enhanced signal transduction processes in astrocytes (Robinson et al., 1999a). Enhanced levels of IL1 and IL6 cytokines are noted near the plaques (Rogers et al., 1996; Cotman et al., 1996) and these factors are expected to mediate the enhancement of signal transduction processes. Activation of signal transduction processes involves enhanced activation (phosphorylation) of MAP kinases. Our research effort has provided a clear demonstration that enhanced signal transduction processes occur in cells surrounding the β-amyloid plaques in affected regions of AD brain (Hensley et al., 1999). We found that activated p38 was readily apparent in neurons and glia surrounding senile plaques in the AD brain. Very little if any p38 activation was found in comparable regions of age-matched control brains or in the cerebellum of AD brains. These results provided the first demonstration of p38 activation in human tissue and definitely show enhanced signal transduction processes in cells near the senile plaques in the AD brain.

P38 MAP kinase and excess nitric oxide synthase

p38 is a redox-sensitive MAP kinase (Abe et al., 1996; Huot et al., 1997). p38 activation plays a role in apoptosis and/or inflammation processes depending on the cell type. p38 is involved in apoptotic processes which are triggered in PC12 cells by deprivation of nerve growth factor (Monti et al., 1996). p38 is also involved in apoptosis in human fibroblasts (Schwenger et al., 1997). On the other hand, inhibitors of p38 prevent the biosynthesis of TNFα and IL1 in

stimulated monocytes (Ridley et al., 1997). Genes induced via the p38 kinase cascade pathway are probably very important in neurodegenerative processes. It was noted that p38 was activated in the hippocampus of gerbils 4 days after a global brain stroke was administered to these animals (Walton et al., 1998). The hippocampus is the area of the brain most susceptible to tissue injury in these animals and the brain region producing the most ROS following a global stroke (Cao et al., 1988; Carney et al., 1992). It has been shown, using inhibitors, that p38 activation is on the pathway to mediating the induction of iNOS in mouse astrocytes (Da Silva et al., 1997) and in rat glia cells (Bhat et al., 1998). Pertinent to the importance of iNOS expression and excess NO formation in stroked brain, Iadecola's group have shown that enhanced iNOS expression occurs after cerebral ischemia in rat (Iadecola et al., 1995a) and that administering catalytic inhibitors of iNOS afforded some protection from the tissue injury caused by a stroke (Iadecola et al., 1995b). PBN has been shown to prevent the induction of iNOS in a mouse septic shock model (Miyajima and Kotake, 1995).

Historical observations on neuroprotective activity of PBN in stroke

PBN has neuroprotective activities in several experimental models. We have reviewed the research in this field (see references Floyd, 1997; Hensley et al., 1996, 1997; Floyd, 1999b). The neuroprotective activity of PBN was discovered serendipitously. Utilizing the gerbil global stroke model, we attempted to make use of PBN to trap and identify free radicals during the reperfusion phase. In previous experiments, we had demonstrated using salicylate trapping that enhanced hydroxyl free radicals were formed during the reperfusion phase of stroke (Cao et al., 1988; Carney et al., 1992). PBN had been used for several years in analytical chemistry experiments to "spin-trap" and identify free radicals in chemical reactions (Janzen and Blackburn, 1969). It had also been demonstrated to be useful to trap certain free radicals in biochemical (see references Poyer et al., 1978; Poyer et al., 1980) for example) and biological systems (see references Bolli et al., 1988; Lai et al., 1979; Lai et al., 1986 for example). Our intent was to use it to see if we could elucidate the free radicals involved in experimental stroke. We found that PBN was an ineffective trap for the free radicals formed in the gerbil stroke model (Oliver et al., 1990), but discovered that it protected the gerbil from death caused by the stroke (Floyd, 1990). This observation has been replicated by other laboratories (Clough-Helfman and Phillis, 1991; Phillis and Clough-Helfman, 1990a, 1990b) and in fact PBN was shown to be neuroprotective (as assessed by brain necrosis) even if administered up to 1 hour after brain reperfusion in the gerbil model (Phillis and Clough-Helfman, 1990a). The results have since been extended to the rat middle cerebral artery occlusion (MCAO) model where PBN was shown to protect the affected brain region even if delivered up to 3 hours after the start of reperfusion (Zhao et al., 1994). A 2,5- disulfonyl PBN derivative, in development for the treatment of stroke, has also been shown to be active in the MCAO model if delivered 2 hours after the start of reperfusion

(Kuroda et al., 1999). It should be noted that Beal's group have shown considerable efficacy of PBN and its 2-sulfonated derivative (S-PBN) in several experimental models of neurodegeneration (Schulz et al., 1995). These include neuroprotective activities of S-PBN in excitotoxicity models using NMDA, KA and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid. Striatial lesions caused by MPP^+, malonate and 3-acetylpyridine were significantly inhibited by PBN as well as by S-PBN.

Chronic dosing of PBN conditioned the old brain to be less susceptible to stroke

We found that old gerbils (15–18 months old retired male breeders) were much more susceptible to a global stroke than were young (3-month-old males) gerbils (Floyd, 1990). If PBN was administered at a chronic low dose (30 mg/kg-day, twice daily) for 14 days to the old gerbils and then its administration ceased, the old treated gerbils were more resistant to a stroke, in fact nearly as resistant as were the young gerbils. This enhanced protection from stroke remained with time after ceasing PBN administration but declined to nearly 30% at 5 days (Floyd and Carney, 1996). The normal enhanced susceptibility of the old gerbils to a stroke returned by 14 days after ceasing PBN administration. There is very little chance that residual PBN remained in the dosed animals for very long after cessation of its administration, for its half-life is 132 minutes (Chen et al., 1990). Therefore, we have concluded that PBN administration mediates the alteration of the old brain such that it becomes more resistant to stroke (Floyd and Carney, 1996). In concert with this notion is the observation that chronic PBN administration lowered the normally age-enhanced oxidized protein levels in old gerbil brain back down to that noted in young gerbils (Carney et al., 1991; Floyd and Carney, 1996). Cessation of PBN administration resulted in the subsequent rise again of the oxidized protein levels in old gerbils back to the original enhanced levels (Carney et al., 1991). We also found that the enhanced behavioral errors of the older gerbils, as compared to younger gerbils, were largely reversed by the chronic 14-day PBN administration. Behavioral errors were assessed by a radial arm maze.

Neuroprotective activity of PBN is not due to its free radical trapping activity

The mechanistic basis of the neuroprotective activity of PBN has not been completely resolved. The discovery in 1969 of the mass action type reaction of PBN with free radicals made it a very useful tool to characterize free radical intermediates in analytical chemistry (Janzen and Blackburn, 1969). However, it is very clear that its neuroprotective action is not due to its ability to trap free radicals in the conventional mass action "spin-trap" mode (see our reviews Floyd, 1996; Hensley et al., 1997; Floyd, 1999b). One main reason is the fact that PBN acts to protect in stroke when delivered up to several hours

after the ischemic/reperfusion event. This means that it was not even present when the most rapid burst of free radicals occurred. The most rapid burst of free radicals in the stroked brain starts almost immediately after starting reperfusion (Cao et al., 1988; Carney et al., 1992). PBN is neuroprotective even if administered up to 3 hours after the start of reperfusion (Zhao et al., 1994). This is a very strong argument against its direct scavenging of ROS as the mechanistic basis of neuroprotective activity of PBN in the stroke model. Additionally, the fact that PBN is very active at chronic, very low levels in mediating a decrease in oxidized protein in old brain argues that its action is not merely mass action in the simple sense of the concept. Significant protein oxidation decreases have been noted in old gerbil brain after administering as little as 1 mg/kg-day PBN for 14 days (Floyd and Carney, 1996). Since PBN distributes essentially equally to all tissues within 20 minutes after its injection (Chen et al., 1990), then the maximum level of PBN that is expected to reach the brain 20 minutes after a 1 mg/kg injection is less than 1 μmolar. In chemical and biochemical experiments where the mass action type free radical trapping activity of PBN is utilized, it is normally used at 10–100 mM; and then it is assumed that it does not trap all of the free radicals present. In stroke experiments where it is administered as a bolus at 100 mg/kg 2–3 hours after reperfusion then the extracellular brain levels was shown by microdialysis to be at most 500 μM (Cheng et al., 1993). Therefore, it is not conceivable that the biological activity of PBN depends upon its classical mass action-trapping activity as noted in chemical systems. In fact, when compared to butylated hydroxytoluene (BHT) or Vitamin E its ability to shut down lipid peroxidation in rat liver microsomal systems, PBN is about 1,000-fold less active than BHT or Vitamin E (Janzen et al., 1994). Therefore, it is not even a very good antioxidant, the potency of which depends upon its ability to trap free radicals.

Behavioral deficits in brain aging/PBN effect

Arendash's group has demonstrated that aged 24-month old rats treated for 4–5 months with a combination of established antioxidants (PBN, vitamin E, and vitamin C) show improved learning and memory retention in the Morris water maze compared to aged controls (Socci et al., 1995). In a follow-up study, they injected aged 24-month old rats with PBN daily (32 mg/kg, ip) for up to 9.5 months (Sack et al., 1996). Several months into the treatment, Morris water maze testing revealed that PBN- and vehicle-treated rats had similar learning in this task. However, PBN-treated aged rats showed remarkably higher memory retention in the water maze compared to controls. In later one-way active avoidance testing, these same PBN-treated animals showed significantly greater learning than controls. These findings, in addition to an earlier study reporting PBN-induced enhancement of radial maze performance in aged gerbils (Carney et al., 1991), clearly demonstrate a cognitive-enhancing ability of PBN in aged rodents. Moreover, the PBN study (Sack et al., 1996) showed that the same group of PBN-treated animals that exhibited

cognitive enhancement also had reduced lipid peroxidation levels (as indexed by TBAR formation) in brain areas important for cognition. Results from other laboratories are consistent with several conclusions from our PBN studies. First, 14-day administration of PBN to accelerated senescence mice resulted in cortical synaptosomes showing EPR spectra indicative of less oxidative stress (Butterfield et al., 1997). Second, daily PBN injections given to accelerated senescence mice beginning in adulthood induced a 1/3 extension in average lifespan (Edamatsu et al., 1995) and PBN given in drinking water to aged mice significantly extended both average and maximal lifespan (Saito et al., 1998).

Hypothesis to explain the neuroprotective activity of PBN

We hypothesize that most, if not all, of the neuroprotective activity of PBN can be accounted for by its ability to suppress signal transduction processes, which can become exacerbated in the brain when it is suffering from any number of insults or "abnormal conditions". For the purposes of illustration, we consider three general "abnormal" conditions that a brain may experience where enhanced signal transduction processes and enhanced oxidative damage are known to occur. The three general "abnormal" conditions are: A) experiencing a large rapid insult, B) undergoing a constant, slowly accelerating-localized smoldering insult and C) experiencing a very low level constant chronic stress. The brain conditions, which generally fit these three categories, are stroke, Alzheimer's disease and an advanced aging brain, respectively. These general concepts are illustrated in Fig. 1. Clearly the conditions apply to specific brain regions for each condition. Figure 2 illustrates the production of "toxic gene products" that are formed at higher levels under each of the three

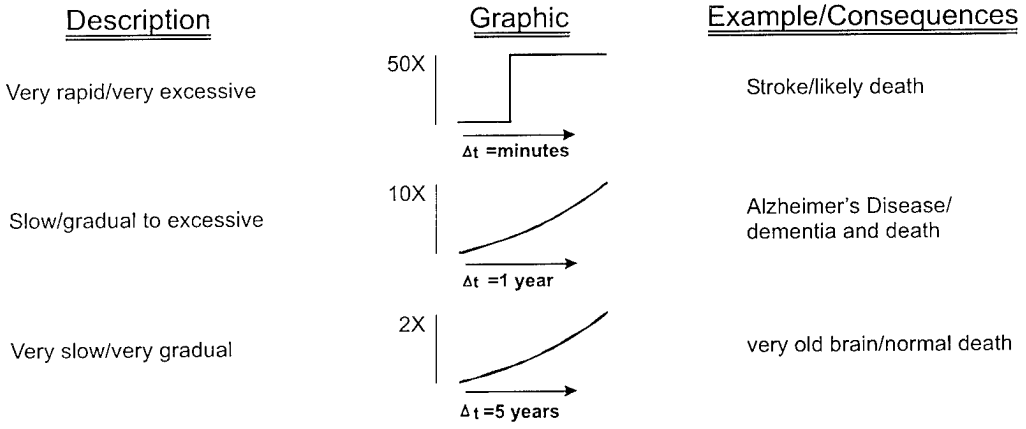

Fig. 1. Representation of brain oxidative challenge states. Particular attention should be directed to the degree of oxidative challenge (ordinate) which is very different in each of the cases and the time-frame (abscissa) which is also very different depending on each of the cases

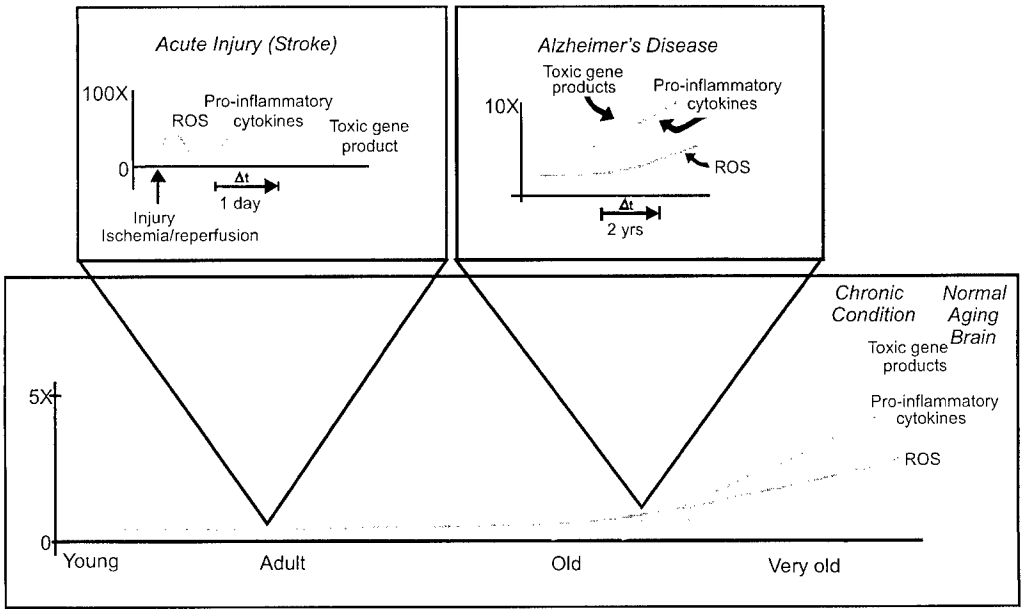

Fig. 2. Illustration of the reactive oxygen species (ROS) expected and the pro-inflammatory cytokine level and toxic gene product levels expected in a coordinated time dependent fashion. The time-frame and levels of each of the species are different in each of the conditions

conditions. The general term "toxic gene products" refers to neurotoxic compounds produced by genes that are induced or are generally upregulated by the insults or abnormal conditions that challenge the brain. We hypothesize that PBN suppresses the production of toxic gene products by suppressing the exacerbated signal transduction processes that leads to the induction of genes that form the neurotoxic products. Perusal of Fig. 2 illustrates that there is a lag time after a stroke before the gene induction processes begin and therefore, if PBN is available during this lag time, then it is expected to mediate the suppression of gene induction initiated by the stroke. In the case of the advanced aging brain, much lower levels of pro-inflammatory cytokines and other activation factors are present when compared to a stroked brain. Nevertheless the amount of cytokines present is higher than in a younger brain. The higher levels of pro-inflammatory cytokines and other factors cause the brain to experience enhanced oxidative stress over a long period of time. We postulate that this leads to enhanced protein oxidation and, for some unknown reason, the brain becomes more susceptible to a stroke. In the case of the advanced aging brain, it is then expected that chronic administration of PBN would suppress the low-grade signal transduction processes and hence lower the amount of oxidized protein. This then positions the brain to become less sensitive to a stroke. This model would then explain the results we have obtained with the stroked gerbils (Floyd, 1990) and the results Siesjo's group obtained in the rat MCAO stroke model (Zhao et al., 1994). This model would also explain the results we obtained with chronic administration of

PBN to the old gerbils (Carney et al., 1991; Floyd, 1990; Floyd and Carney, 1996). Based on this model to explain the results in the old gerbils and in the stroked brain, we think that the Alzheimer's brain suffers a condition that is represented as an intermediate somewhere between the two extremes of stroke and the advanced aging brain (see Fig. 1 and Fig. 2).

Utilizing the logic of this model we hypothesize that chronic PBN administration will suppress the enhanced signal transduction processes in the Alzheimer's brain and hence significantly lower the production of toxic gene products and decrease the amount of oxidized protein. We consider that dementia is due in part to damaged neurons caused by the production of "toxic gene products" which are made as a result of enhanced neuro-inflammatory processes that are triggered by β-amyloid plaques. Chronic PBN treatment is expected to decrease neuro-inflammatory processes and therefore, should be able to decrease dementia. It is possible that chronic PBN administration, perhaps for a relatively short period of time, may restore most of the normal functioning of the brain. If this hypothesis is valid, it is expected that PBN would have no influence on β-amyloid deposition. Therefore, the triggering stimulus would still be persistently present and hence, cessation of PBN administration would then result in the restoration of the neuro-inflammatory processes leading to enhanced protein oxidation and eventually to the redevelopment of dementia.

PBN inhibition of signal transduction processes

Our interest in signal transduction processes as the possible site of action of PBN became more intense as more and more evidence accumulated showing that ROS is involved in some fashion in signal transduction processes (see Reference Suzuki et al., 1997 for a review). There are many published reports now demonstrating that PBN suppresses signal transduction processes both in cultured cell systems as well as in animal models. The first demonstration of this fact was made evident in the stroked gerbil brain (Carney et al., 1994), where it was noted that PBN administration suppressed the induction of several genes. A more clear-cut example was then made by Miyajima and Kotake (Miyajima and Kotake, 1995) who demonstrated that PBN inhibited the induction of iNOS in the liver of a septic shock model, i.e. LPS-treated mice. They demonstrated that PBN inhibited iNOS induction but that it did not act as a catalytic inhibitor of the fully expressed and functional iNOS enzyme. Utilizing a multiprobe ribonuclease protection assay we have shown in the rat LPS-induced septic shock model that PBN suppresses a wide array of genes induced in liver (Stewart et al., 1999). Utilizing a neonatal rat model of AIDs Dementia Complex where gp120, the HIV envelope protein, is administered we demonstrated that PBN prevented the gp120-induced production of NO in the neonatal rat brain (Tabatabaie et al., 1996). Our interpretation of the results was that PBN prevented the induction of iNOS in the brain. Kotake's laboratory has recently demonstrated that PBN prevents the enhanced synthesis of NO in brain induced by a direct brain injection of LPS as an experimental model of bacterial meningitis (Endoh et al., 1999). In

cellular systems, Kotake's group has shown that PBN at higher levels inhibits LPS-mediated upregulation of iNOS and COX-2 in a macrophage cell line (Kotake et al., 1998). PBN prevented the LPS-mediated NFκB movement to the nucleus. PBN at higher concentration inhibited catalytically the expressed iNOS enzyme but did not act catalytically on the COX-2 enzyme (Kotake et al., 1998). Our group has examined the efficacy of PBN in a series of experiments involving signal transduction processes in cultured rat astrocytes. The assays have focused on p38 activation processes in the astrocytes. The results (Robinson et al., 1999a, 1999b) can be summarized as such: A) astrocytes are activated by various cytokines especially IL-1β and H_2O_2 and B) PBN as well as N-acetylcysteine (NAC) suppresses IL-1β and H_2O_2 mediated p38 activation. In this system, p38 is at first rapidly activated and then is subsequently shut down in a biphasic response. It should be noted that synthesis of cytokines is triggered in the activated cells and that PBN suppresses this.

Does PBN suppress neuro-inflammatory processes in vivo?

The previous sections provide background information which clearly implicate that PBN would be expected to suppress neuro-inflammatory processes. Prior to now, no experiment has ever been set up to directly test this notion in an in vivo model. We report here results of an experiment clearly showing that PBN does suppress signal transduction events linked to neuro-inflammatory processes in a KA — brain damage model in rats. Although the KA model is not a classical neuro-inflammatory model in the sense that AD would be, it nevertheless does provide very valuable information and surprises.

We have utilized the KA model of epilepsy, where a single systemic dose of the excitotoxin initiates a process of hippocampal neurotoxicity (Bernard and Wheal, 1995). Rats treated with KA suffer recurrent convulsive seizures and apoptotic neuron loss in the CA1 and CA3 regions of the hippocampus (Pisa et al., 1980; Schwob et al., 1980). Seizure activity is correlated with neuroanatomical changes including mossy fiber sprouting in the dentate gyrus, hippocampal sclerosis, and eventually, neuronal death (Schwob et al., 1980; Sperk et al., 1996; Cronin et al., 1992). The lesions produced by systemic KA treatment resemble those seen in hippocampi of human temporal lobe epileptics (Sommer, 1880; Schwob et al., 1980; Pisa et al., 1980; Sperk et al., 1996). KA appears to act directly on non-NMDA type ionotropic glutamate receptors (Bernard and Wheal, 1995), leading to cell death, which is predominantly apoptotic in nature (Simonian et al., 1996; Bengzon et al., 1997; Yang et al., 1997; Cheung et al., 1998). Our goal was to use KA to chronically stimulate signal transduction pathways and determine if PBN administration would suppress these events.

Materials and methods

Animals. Adult male Sprague Dawley rats (225–250 g each) were injected subcutaneously behind the neck with KA (Sigma Chemical, St. Louis MO) at a dose of 10 mg/kg, or with

vehicle alone (saline). Animals were observed for 4 hours following KA treatment and seizure activity was rated according to the scale developed by Racine et al. (1972) and modified by Mathis and Ungerer (1992). Briefly, seizure severity was scored in five stages; from Stage 1 where animals had mild myoclonus with moderate jerking movements of one or two links to State 5 where animals had status epileptic, i.e. continuous seizure activity for 30 minutes or longer with explosive jumps.

Phenyl-N-*tert*-butylnitrone was synthesized at the Oklahoma Medical Research Foundation (Oklahoma City, OK) and was injected at a dose of 150 mg/kg intraperitoneally, in saline vehicle, 90 minutes after KA treatment. The 150-mg/kg bolus of PBN is a standard dose and has repeatedly been shown effective in rodent models of ischemia-reperfusion injury and sepsis, which causes no obvious side effects such as lethargy and hypothermia that, can sometimes be seen at higher doses (Hensley et al., 1997).

Immunohistochemistry

For immunocytochemical studies, animals were anesthetized with pentobarbital and perfused with saline followed by 4% paraformaldehyde in saline. Brains were sectioned into 30 μm slices, which were incubated in 4% normal goat serum in saline for 30 min. at ambient temperature. After three washes with saline, the sections were incubated overnight at 4°C in saline plus 0.025% triton X-100, 1% goat serum, and primary antibody. Immunoreactivity was visualized by the avidin-biotin-bridged immunoperoxidase method using 3,3′-diaminobenzidine (DAB) as the chromagen (Hsu et al., 1981). The anti-phospho-p38 antibody was an affinity-purified rabbit IgG purchased from New England Biolabs (Beverly, MA), used at 1/300 dilution. Affinity purified rabbit IgG antibodies against c-Fos, c-Jun and the p65 subunit of NFκB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1/1,000, 1/1,000, and 1/300 dilution, respectively. Photomicroscopy was performed on a Zeiss Axioplan 2 spiker instrument (Carl Zeiss Inc., Thornwood, NY).

Electromobility gel-shift assays (EMSAs)

EMSAs were conducted to determine binding of activated NFκB complexes to synthetic oligonucleotide consensus sequences. The NFκB-binding oligomer was a 22-mer: 5′-GATCGAGGGGACTTTCCCTAGC-3′, purchased from Stratagene (La Jolla CA). Double-stranded oligomers were labeled with [γ-^{32}P]ATP using 10 u/reaction of T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH). Hippocampi were dissected free and homogenized, and nuclear protein extracts were prepared as described (Sonnenberg et al., 1989). Binding reactions (30 μL) were performed at room temperature in reaction mixtures containing 40 μg protein, 20 mM Tris-HCL pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 100 μg/mL sonicated salmon sperm DNA, 10% glycerol, and approximately 0.2 ng (50,000 cpm) of the specific probe. Protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels run at 150 V

in 50mM Tris/50mM boric acid/1mM EDTA. Gels were then dried and autoradiographed overnight.

Terminal deoxyuridine nick-end labeling (TUNEL)

DNA fragmentation characteristic of apoptosis was visualized by 3′end labeling with biotin-derivatized deoxynucleotides via terminal deoxynucleotidyl transferase catalysis. A commercially available TUNEL kit was used (TdT FragEL, Calbiochem, San Diego CA). Biotinylated nucleotides were detected using streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Hsu et al., 1989). Tissue sections thus labeled were counterstained with methyl green as an aid to morphological evaluation.

Ribonuclease protection assays

Approximately 100mg of hippocampal tissue was homogenized in trizol isolation reagent (Life Technologies, Gaithersburg, MD) using a Dounce-type homogenizer. Total RNA in the extract was quantified by UV absorbance at 260nm. Inflammation and apoptosis-associated mRNA species were selectively visualized using a multiprobe ribonuclease protection assay (RPA). Radiolabeled probes were synthesized from DNA templates containing a T7 RNA polymerase promoter (Pharmingen, San Diego, CA). Templates were transcribed in the presence of [γ-^{32}P]ATP to yield radioactive probes of defined size for each mRNA. Probes were hybridized with total hippocampal RNA, then samples were treated with RNAse A and T1 to digest single-stranded RNA. Intact double-stranded RNA hybrids were resolved on 5% polyacrylamide/8M urea gels to produce bands detected by autoradiography.

Results

Beginning approximately 30 minutes after KA injection, animals displayed archetypical epileptiform behavior including "wet dog" shakes, facial clonus, nodding, and forelimb clonus. Three hours after injection, KA-treated rats showed full limbic motor seizures including rearing and loss of postural control, as well as hypersalivation, circling and jumping. Rats treated with PBN 90 minutes after KA injection did not develop full limbic seizures by the 3-hour time point (Table 1). Moreover, PBN rescued the KA-treated animals from mortality when evaluated at the end of the four-day experiment (Table 1). No behavioral, physiologic or histologic alterations were observed in animals receiving PBN only.

The first immunochemical analysis of KA-treated rats was aimed at determining whether PBN could antagonize the AP-1 system in vivo. Immunocytochemical analysis was performed using well-characterized antibodies

Table 1. Suppression by PBN of limbic seizures and mortality in kainic acid-treated rats. Seizure activity was ranked on a five-point scale as described in the methods

Treatment	Seizure intensity	Mortality (4 days)
Kainic acid (N = 30)	4.9 ± 0.4	12/30 (38%)
Kainic acid + PBN (N = 20)	2.3 ± 0.3*	0/20 (0%)**

*$P < 0.05$ (Student's t-test)
**$P < 0.02$ (χ^2 test)

against the two AP-1 subunits, c-Fos and c-Jun. Within hours of KA treatment, c-Fos and c-Jun expression increased in hippocampal neurons, particularly within the CA1 and CA3 regions. The c-Fos and c-Jun expression was maintained throughout the four-day experiment (not illustrated), consistent with previously reported data (Bing et al., 1997). A single injection of PBN completely suppressed c-Jun expression in both CA regions and in the dentate gyrus while c-Fos expression was suppressed by PBN only in the CA1 and CA3 regions, where most of the pathological changes were manifest (data not shown). We also have done an immunohistochemical analysis of p38 activation using an antibody specifically directed against the dual-phosphorylation motif, which is present only on the active p38 kinase (Raingeaud et al., 1995). Within 4 hours of KA treatment, p38 activation was seen within the hippocampus in a pattern consistent with that of AP-1 activation (Fig. 3). As in the case of AP-1, PBN suppressed p38 phospho-activation (Fig. 3). The p38 system remained activated somewhat above the level of controls at the four-day timepoint, but this chronic activation was not as dramatic as in the AP-1 case (data not shown).

The NFκB transcription factor is also ubiquitously activated by physiologic stress and may potentiate excitotoxic damage in striatal neurons (Qin et al., 1998). Alternatively, NFκB seems to serve a protective role in hippocampal neurons undergoing an oxidative insult (Mattson et al., 1997) and may actually play an antiapoptotic role in TNFα-stimulated cells (Van Antwerp et al., 1996; Wang et al., 1998). NFκB is part of a signal transduction cascade, which has traditionally been thought of as distinct from the Jnk and p38 cascade modules, though correlated activation of the three pathways is often noted in cell culture experiments. Several lines of evidence now suggest that p38 and other MAPK enzymes may hyperactivate NFκB (reviewed in Schulze-Osthoff et al., 1997), while inhibition of p38 can suppress transactivational potential of NFκB (Vanden Berghe et al., 1998). We therefore sought to determine whether NFκB was activated by KA in a PBN-sensitive manner. NFκB activation can be indexed several ways. Immunologically, NFκB activation can be inferred from increased immunoreactivity of an epitope on the p65 subunit, which is exposed upon NFκB recruitment (Rice and Ernst, 1993). As shown in Fig. 4, NFκB-p65 immunoreactivity in the hippocampus increased dramatically within hours of KA treatment, and this effect was suppressed by PBN. The immunochemical data was corroborated

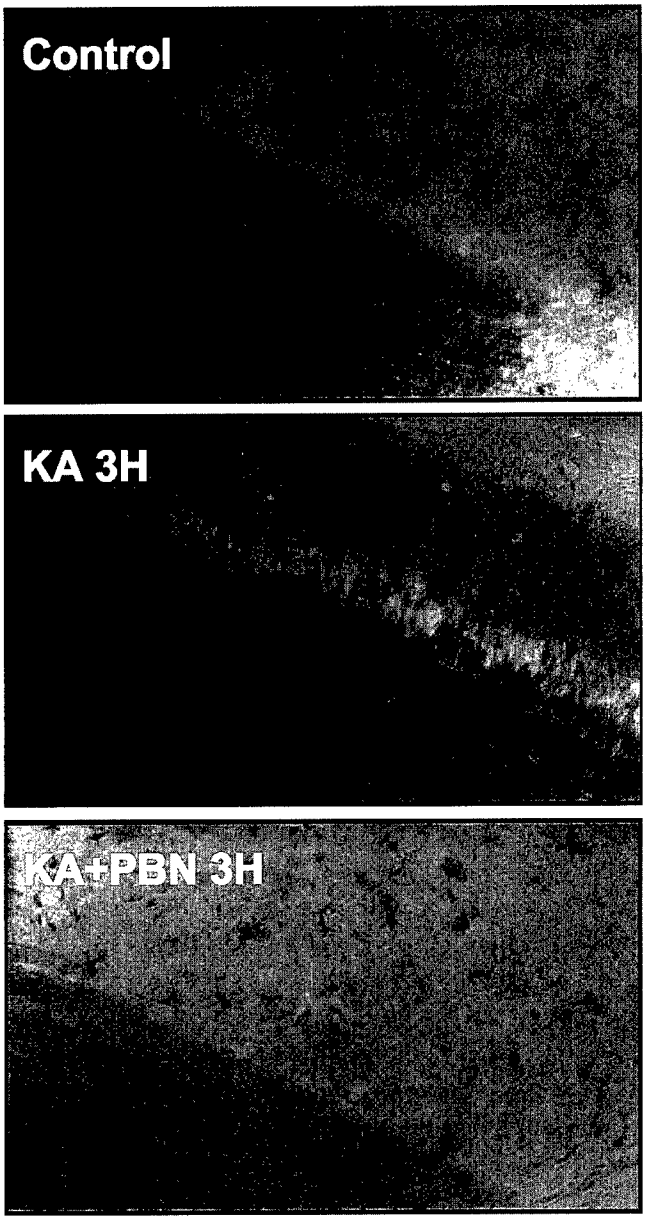

Fig. 3. Kainic acid increases p38-MAPK activation in the hippocampus as indicated by increased phosphorylation of the p38-MAPK activation domain. The CA1 subregion is depicted. Immunohistochemistry was performed using an antibody directed against the phosphorylation domain of the active p38 MAPK enzyme ($pThr^{180}$-Gly^{181}-$pTyr^{182}$)

by EMSA analysis, which showed a dramatically increased NFκB binding activity in hippocampal nuclei of KA, treated rats, which was partially mitigated by PBN cotreatment (Fig. 4).

Hyperactivation of the Jnk, NFκB and p38 signal transduction pathways could be anticipated to have numerous detrimental consequences. All three signaling pathways have been linked to transcription of inflammatory

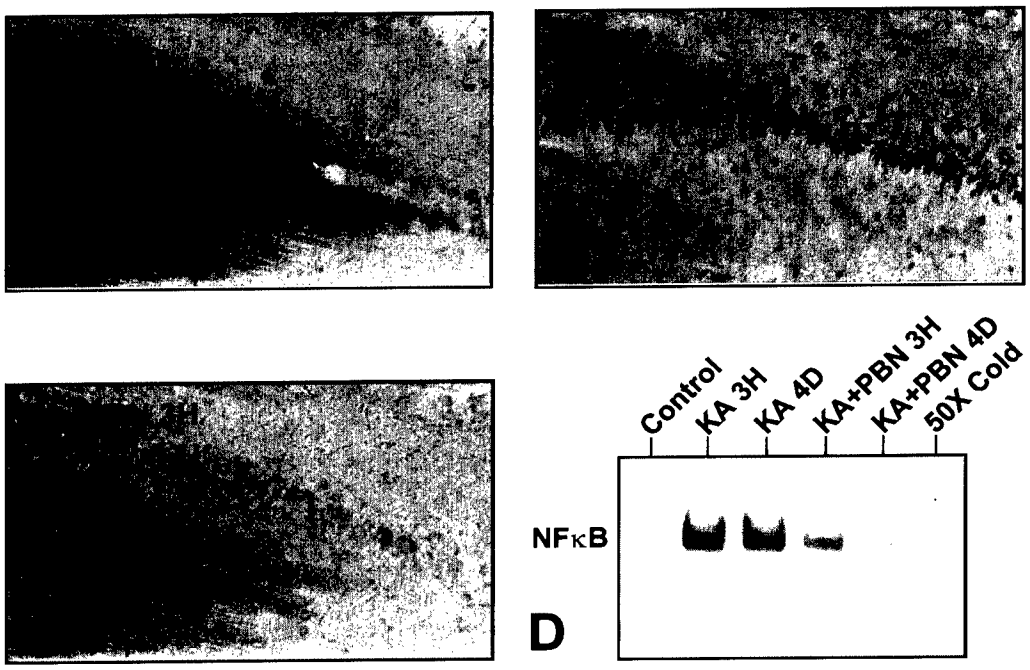

Fig. 4A–D. Kainic acid increases NFκB activation in the hippocampus. **A**, **B**, and **C** illustrate exposure of the p65 subunit of the NFκB complex following KA treatment (arrows). **D** Electromobility gel shift assay demonstrating increased NFκB binding activity in nuclear extracts induced by KA treatment and suppressed by cotreatment with PBN. Specificity of binding was evidenced by competition for the NFκB complex by an unlabeled (cold) oligonucleotide probe (rightmost lane)

cytokines and to modulation of apoptosis (Kawasaki et al., 1997; Kummer et al., 1997; Yang et al., 1997; Qin et al., 1998). We therefore sought to determine whether cytokine and proapoptotic genes were being transcribed at a greater rate in the KA treated rats than in normal rats, and whether PBN could abrogate such an effect. Using a multiprobe ribonuclease protection assay, several inflammatory cytokines were clearly found to be transcribed following KA treatment (Fig. 5). IL1α, IL1-β, IL-6 and TNF-α transcription were strongly induced by KA. Within the timeframe that cytokine transcription was enhanced, several proapoptotic genes were also induced. Most notably, the Fas antigen mRNA was strongly induced following KA and this elevation was maintained for at least four days (Fig. 6). PBN treatment suppressed transcription of both inflammatory cytokine gene products and proapoptotic gene products while having minimal effect on transcription of constitutively-expressed "housekeeping genes" including the L-32 ribosomal mRNA and glyceraldehyde phosphate dehydrogenase mRNA (Figs. 5, 6). PBN suppression of cytokine mRNA transcription was relatively unspecific. Interestingly, PBN displayed particular potency in suppressing Fas antigen and caspase 3 transcription, while other apoptosis-associated mRNA species analyzed by RPA were somewhat less affected by the nitrone (Fig. 6).

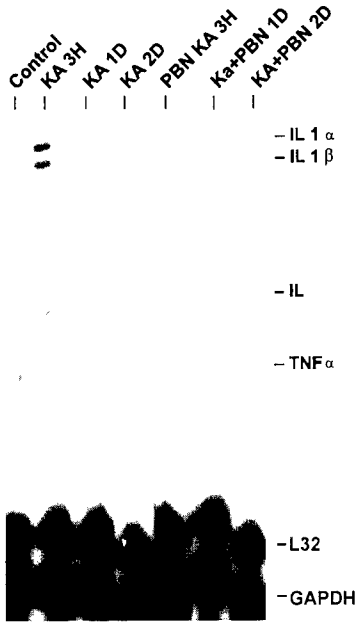

Fig. 5. Kainic acid stimulates the transcription of proinflammatory cytokines in the hip pocampus as determined by multiprobe ribonuclease protection assay (RPA)

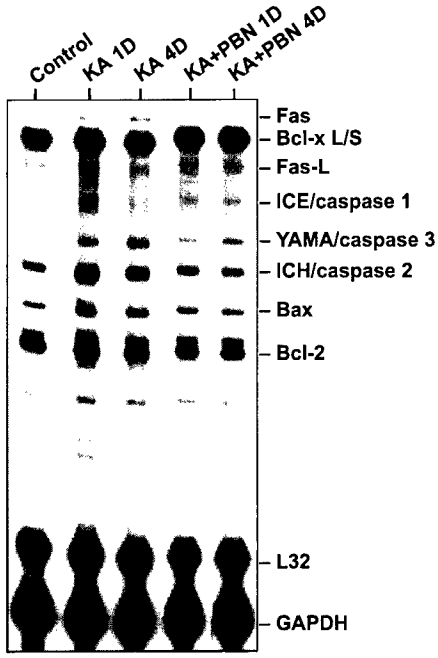

Fig. 6. Kainic acid stimulates transcription of proapoptotic genes in the hippocampus as determined by multiprobe ribonuclease protection assay (RPA)

As a final indication of KA-induced hippocampal damage, in situ TUNEL staining was performed to assess frank apoptosis. KA treatment caused DNA damage indicative of an apoptotic process within four days of subcutaneous administration (data not shown). Apoptosis was largely restricted to the CA1 and CA3 regions of the hippocampus wherein c-Fos was most strongly expressed. Administration of PBN 30 minutes after KA exposure strongly inhibited this apoptosis as indicated by diminished TUNEL staining in hippocampi from PBN treated animals. TUNEL staining for apoptotic nuclei therefore corroborates the pattern of KA-induced and PBN-sensitive immediate early gene expression, and the pattern of proapoptotic gene induction illustrated in Fig. 6.

Discussion

The results of the KA induced brain damage experiment highlights several important points. These include: A) the clear demonstration of the neuroprotective activity of PBN in the KA-induced epilepsy model and B) the potent activity of PBN in suppressing signal transduction processes in the three MAP kinase pathways (AP-1, NFκB and p38) in an in vivo model where excitoxicity and apoptosis have already been implicated. This suggests an inhibition of these three pathways by the experimental compound phenyl-*tert*-butylnitrone was associated with diminished cytokine elaboration, prevention of neuronal apoptosis, reduced seizure activity, and reduced mortality. While the AP-1, NFkB, and p38 pathways are known to respond positively to oxidants and negatively to antioxidants in cell culture (Suzuki et al., 1994; Guyton et al., 1996; Robinson et al., 1999a), the data in this present study are the first to demonstrate the sensitivity of these three pathways to PBN (sometimes classed as an antioxidant compound) within the context of an established in vivo model of hippocampal neurodegeneration.

The findings of the present study extend upon previous observations concerning the broad-spectrum neuroprotective action of nitrone compounds, and provide a novel context for discussing the pathology of excitotoxicity. PBN and related nitrones have been shown to suppress striatal excitotoxic lesions induced by NMDA, KA, and AMPA, though not by virtue of any obvious direct interaction with glutamate receptors (Shultz et al., 1995). Similarly, PBN and a sulfated analog inhibit striatal lesions caused by mitochondrial inhibitors such as malonate and the 1-methyl-4-phenylpyridinium (MPP+; Shultz et al., 1995). Nitrones suppress apoptosis and oxidative stress in cultured Down's syndrome neurons (Busciglio and Yankner, 1995), and similarly inhibit chemically induced thymocyte apoptosis in vitro (Slater et al., 1995), though the influence of nitrones on apoptosis in vivo has not been well studied. Unfortunately, the pharmacologic effects of nitrones in most previous investigations were not correlated with biomarkers of oxidative stress, inflammation or apoptosis. The present data suggest that suppression of apoptosis by PBN in the KA model and possibly other models of neurodegeneration is likely due to mitigation of proinflammatory or

proapoptotic gene expression under the control of the AP-1, NFκB, and p38 MAPK pathways. While the ultimate cellular target(s) for PBN action remain unclear, the present data suggest that the broad-spectrum neuroprotective action of the nitrone class of compounds (Hensley et al., 1997) might be due, in part, to antagonism of crucial oxidation-sensitive signal transduction elements linked to the initiation of apoptotic programs.

PBN neuroprotection and future novel therapeutics

The data clearly show that administration of PBN at least 90 minutes after the administration of KA affords significant protection. It is not known the time to give PBN in reference to KA for achieving maximum efficiency. However, in preliminary experiments, we noted a lack of protection and in fact, perhaps an enhancement of KA toxicity if PBN was given 30 minutes prior to giving the toxin. It is possible in this case that PBN perhaps inhibits metabolic processes whereby KA is rendered inactive, although this has not been studied. The fact that PBN was effective after the KA administration, again as in the case of stroke, indicates that an insult to the brain sets off processes which require some time to reach their full destructive potential. Much evidence in the case of stroke, and now as we have presently demonstrated in the KA model, suggests that signal transduction processes lending to gene induction is a requisite to begin the events leading to brain injury. Agents, such as PBN, which interfere or suppress these processes occurring during the lag phase, may be good candidates for therapeutics of several neurodegenerative diseases.

In the case of Alzheimer's disease, we consider the β-amyloid plaques are localized constant trigger centers. Therefore, to suppress this constant stress it requires the constant administration of an agent that would suppress the localized neuro-inflammatory processes. We envision that treatment with the novel therapeutic, based on the notions outlined here, although it probably would not reverse the β-amyloid deposition, it would however ideally suppress the brain damage caused by the neuro-inflammatory processes triggered by the senile plaques. We consider it likely that the dementia associated with AD is the indication that would benefit the most from the novel therapeutics that may be developed based on these concepts. These ideas have yet to be thoroughly tested but do offer a new approach and possibly an inordinate potential for the treatment of several neurodegenerative diseases.

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Cloning and expression of *MP13* gene from rat hippocampus, a new factor related to guanosine triphosphate regulation

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Abstract

C-Fos and the *Fos*-related antigens (*FRA*) are induced by various stimuli. A novel 35–37 kDa FRA was induced much longer after the treatment using kainic acid (KA) and may be very important for neuronal survival after brain damage. To identify this long-term *FRA*, we have constructed a cDNA library derived from hippocampus after KA treatment and screened it with an antibody highly conserved M-peptide region of *FRAs*. One gene, *MP13*, was cloned with a 1662 bp open reading frame and coded for a 554-amino acid protein. *MP13* has a leucine zipper region, a glutamine repeat region, and has high similarity to the activator of the small guanosine triphosphate (GTP)ase *Rab5*. Gel retardation analysis revealed that *MP13* functions as a GTP regulation related factor.

Keywords: *Fos*-related antigen; Transcription factors; Gene; Cloning; Guanosine triphosphate; Immunoscreening; Gel-shift

The induction of c-*Fos* and the *Fos*-related antigens (*FRA*), including *Fos B*, *Fra-1* and *Fra-2*, by various stimuli has been extensively studied [1,3,4,6,9]. In most cases, the induction of FRA are rapid and transient, lasting about 1–4 h after the stimuli [7,8]. However, several laboratories including ours have described a novel 35–37 kDa *FRA* which is induced for a much longer time after stimulation and whose distribution is distinct from the induction of other FRAs. This long-term elevated *FRA* can be induced by the chronic administration of cocaine [6,10] or morphine [6]. We have found that systemic administration of kainic acid (KA), a glutamate analog, induced a 35 kDa FRA that persisted for up to 5 months in the granule cells of the dentate gyrus [1,5,9]. The administration of KA not only caused epileptic seizures but also damaged certain areas of the rat brain, especially the hippocampus where axonal sprouting from the granule cells of the dentate gyrus occurred. The long-lasting changes of *FRA* expression, thus, may underlie these long-term effects of the KA. This long-term expression of *FRA* after KA treatment suggests that the granule cells of the dentate gyrus remain activated for a protracted time, which presumably reflects permanent changes in their genomic programming. The gene encoding for this FRA protein, therefore, may be very important both in the brain's adaptation to cocaine and morphine, and in neuronal survival and sprouting after brain damage.

In order to identify this long-term *FRA*, we have constructed a cDNA library derived from the hippocampus 3 days after KA treatment. One of 41 positive clones, *MP13*, from 2 million screened colonies was selected. We now report the cloning and expression of *MP13*, which may functionally relate to guanosine triphosphate (GTP) regulation.

The KA-treated rat hippocampal cDNA library was constructed with the Lambda ZAP cDNA synthesis kits (Stratagene, La Jolla, CA). Adult male Fischer rats (250–300 g, Charles River, Raleigh, NC) were injected intraperitoneally (i.p.) with KA (8 mg/kg; 1 ml/kg) or saline (control). Only the animals with convulsive behavior (forelimb clonus with intermittent episodes of whole-body clonus) within 4 h following KA administration were used for this study. Rats were sacrificed by decapitation 3 days

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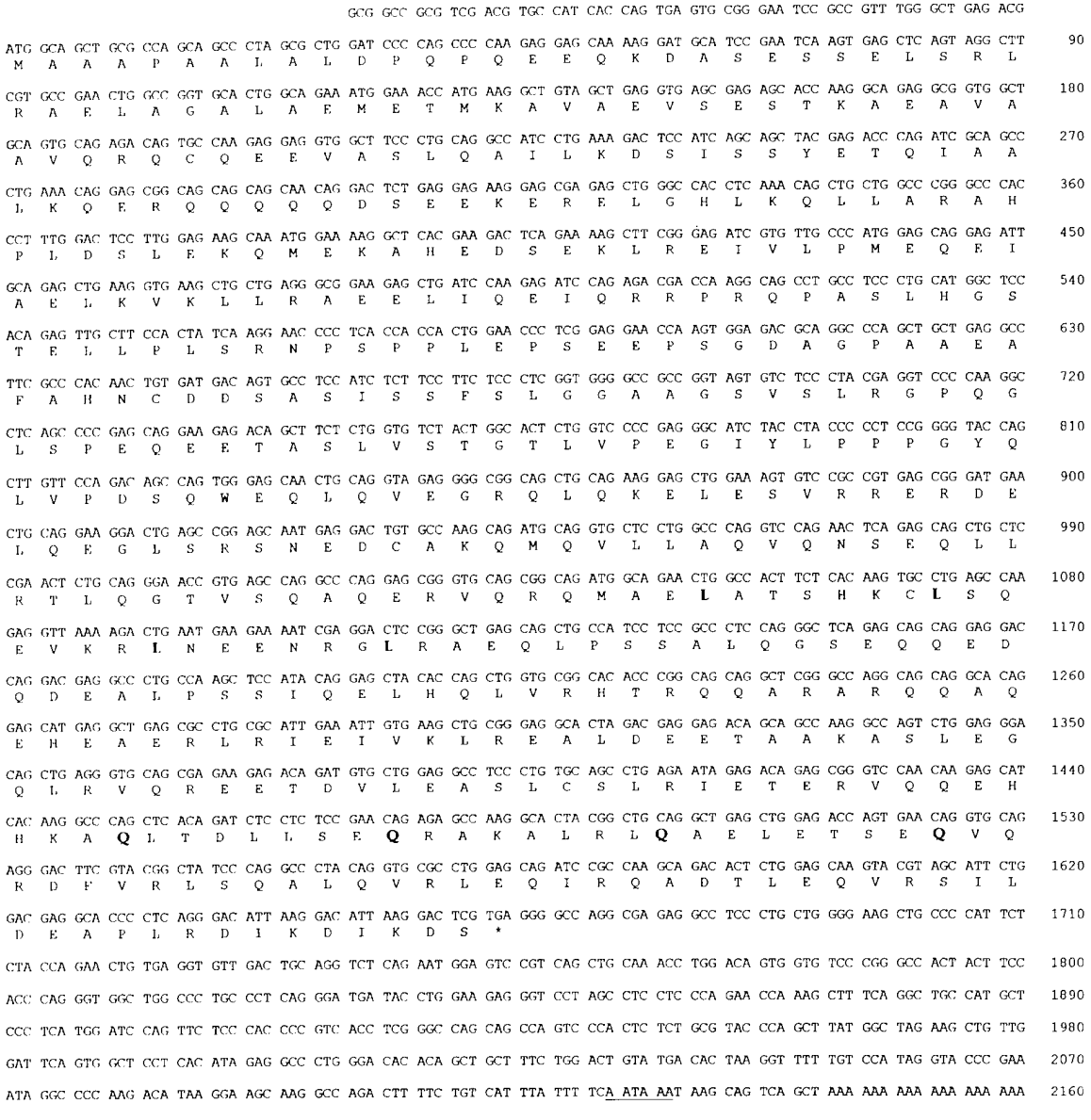

Fig. 1. The complete DNA and protein sequences of *MP13* clone. The cloned cDNA sequence of the *MP13* has 2221 bp, which contains an open reading frame of 1662 bp, a 60 bp 5′-non-coding region, and a 500 bp 3′-non-coding sequence with a poly(A)$^+$ tail. The predicted sequence of the 554 amino acid *MP13* protein is indicated by the single-letter amino acid code. The polyadenylation signal AATAAA is underlined. The heptad leucine repeats and octet glutamine repeats are printed in bold.

after KA treatment, and RNA of hippocampi were extracted with TRI reagent and purified from oligo-dT affinity columns. Five μg of poly(A) + -rich RNA were used to construct the lambda ZAP II cDNA library. The cDNA was synthesized with oligo-dT primer at 37°C for 1 h with SuperScript II reverse transcriptase in 1 × reverse transcriptase buffer, 200 mM dithiothreitol, and 2 mM dNTP. The second strand of the DNA was synthesized at 16°C for 2 h with *Escherichia coli* DNA polymerase and *E. coli DNA* ligase in 1 × second strand buffer and 2 mM dNTP. The synthesized DNA was ligated with *Sal*I adapters first, then digested with *Not*I, size-fractionated, inserted to a cloning vector and packaged in *E. coli* cells.

The *Fos*-related antigen (FRA) gene was screened with the antibody generated against a highly conserved sequence of the M-peptide of the *FRA* [3,6] using the PicoBlue™ Immunoscreening Kit (Stratagene, La Jolla, CA). Colonies were seeded onto LB plates supplemented with 2 mM IPTG and transferred onto a nitrocellulose membrane. The membranes were blocked in a Tris–NaCl/Tween-20 buffer containing 3% skim milk and incubated at 4°C overnight with a 1:1000 dilution of *FRA* antiserum. After washing in

```
          300         310         320         330         340
           *           *           *           *           *
Mp13       DELQEGLSRS NEDCAKQMQV LLAQVQNSEQ LLRTLQGTVS QAQERVQRQM
           ^^^^^^^^^^ ^v^v--^^-^ ^^^^v^v^^^ ^^^ ^^v-v- vv^-^vvv^^
Rabaptin5  eELQqGLSqa krDvqeQMaV LmsreQvSEe LvR-LQkdnd slQgkhslhv
                620         630         640         650         660

          350         360         370         380         390
           *           *           *           *           *
Mp13       AELATSHKCL SQEVKRLNEE NRGLRAEQLP SSALQGSEQQ EDQDEALPSS
           ^vv-^-^vv^ ^^---v^-^v vvvv^ ^^^  v^-vv^^^^v ^^^v-^vvvv
Rabaptin5  slqqaedfiL pdtteaLrEl vlkyR-Edi- invrtaadhv Eeklkaeilf
                670         680         690         700         710

          400         410         420         430         440
           *           *           *           *           *
Mp13       IQELHQLVRH TRQQARARQQ AQEHEAERLR IEIVKLREAL DEETAAKASL
           ^^^vv^vv^v v^^^vv-vv^ v^v^^v-vvv v-^v-^^--^ ^vv---^^v^
Rabaptin5  lkEqiQaeqc lkenleetlQ leienckeei asIssLkaeL erikveKqqL
                720         730         740         750         760

          450         460         470         480         490
           *           *           *           *           *
Mp13       EGQLRVQREE TDVLEASLCS LRIETERVQQ EHHKAQLTDL LSEQRAKALR
           ^^v^^v^-^^ v^v^^ -^ ^ ^-^^-^vv^^ -vv^^v^-^^ ^v^^^-^^v^
Rabaptin5  EstLRcksqq lesLq-ei-k islEeqlkke taaKAtveqL mfEeknKAqR
                770         780         790         800         810

          500         510         520         530         540
           *           *           *           *           *
Mp13       LQAELETSEQ VQRDFVRLSQ ALQVRLEQIR QADTLEQVRS ILDEAPLRDI
           ^^^^^^-^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^v^v^^
Rabaptin5  LQtELdvSEQ VQRDFVkLSQ tLQVqLErIR QADsLEriRa ILndtkLtDI
                820         830         840         850         860

          550
           *
Mp13       KDIKDS*
           ^^^v^^
Rabaptin5  nqlpet
```

Fig. 2. *MP13* peptide sequence was homologous to the C-terminal peptide sequence of rabaptin-5. Peptide homology searching was done with the Protein Identification Resource (PIR) database. M13 protein represents *MP13* peptide sequence; rabapti represents the rabaptin-5 peptide sequence; '∧' indicates the identity between the two amino acid residues; 'v' indicates that there are two different amino acid residues. The numbers indicate the positions of residues in the two sequences.

the Tris–NaCl buffer, the positive colonies were visualized using the alkaline phosphatase method. Forty one positive clones were isolated from 2×10^6 plaques. Polymerase chain reaction (PCR) was performed using primers conserved in all *FRA*. The predicted PCR fragments were found in one clone, *MP13*.

To further identify the clones, the fusion protein of *MP13* was tested by Western blot analysis. *MP13* was only recognized by *FRA* antibody, but not by c-*Fos*, *Fra-1*, or *Fra-2* antibodies (unpublished data). *MP13* and five other selected clones were used for further sequence analysis.

DNA samples for sequencing were prepared with Wizard™ Mini-Preps kits (Promega, Madison, WI) according to the manufacturer's protocol and 1 μg DNA was sequenced by the dideoxynucleotide chain termination method with Sequenase 2.0. DNA homology searching was done in the GenBank database with GCG software and the predicted amino acid sequences were compared with the Protein Identification Resource (PIR) database. *MP13* DNA sequence has been submitted to GenBank nucleotide sequence database with accession number U34932.

A complete cDNA sequence, 2221 bp, was generated from *MP13*. As shown in Fig. 1, the whole sequence contains an open reading frame of 1662 bp, a 60 bp 5′-non-coding region and a 500 bp 3′-non-coding sequence with a poly(A)$^+$ tail. It codes for a protein of 554 amino acid residues. This protein contains a series of four leucine heptad repeats which are separated by 111 amino acid residues from a series of four glutamine octet repeats. *MP13* protein has same peptide sequence except that the 5th amino acid was replaced by a Q (glutamine). The leucine repeats found in *MP13* protein resemble the leucine zipper found in all of known *FRA*s. However, *MP13* protein apparently lacks the basic DNA-binding region in all of known *FRA*s. This result suggests that *MP13* protein has a different function from the common function of the *FRA* protein. The function of four octet glutamine repeats is currently unknown. It warrants further investigation.

The homology searching revealed that two regions of the *MP13* protein sequence are similar to the C-terminus of *rabaptin-5* (Fig. 2), an activator of the small GTPase *Rab5* [12]. The first region extends from peptide position 298–335 and has more than 80% similarity to *rabaptin-5*. This region represents an α-turn point. The second region from peptide position 481 to the end has more than 90% similarity to *rabaptin-5*. Since *rabaptin-5* is a activator of small GTPase, the homologous searching result suggested that the function of *MP13* be related to the GTP regulation.

To identify the function of *MP13*, we translated *MP13* in vitro and tested the translated *MP13* protein in gel mobility-shift assay. The translation products of *MP13* DNA (0.1 μg; Fig. 3, lanes *MP13*) and vector SK (Fig. 3, lane SK) in vitro was generated by the Single Tube Protein™ system (Novagen, Madison, WI) using T3 RNA polymerase. The translation mixtures were analyzed by gel electrophoresis with (Fig. 3, Lanes +*FRA* Ab)/without immunoprecipitation with the *FRA* antibody, and with (Fig. 3, Lane +M

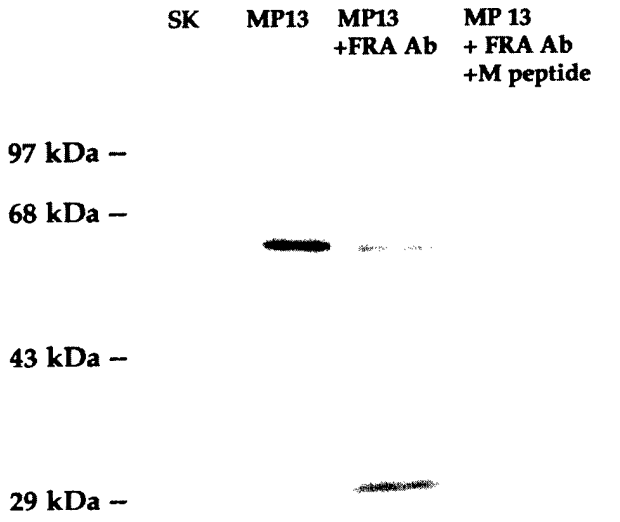

Fig. 3. Translation of *MP13* DNA in vitro. Translation of *MP13* DNA (0.1 μg; lanes *MP13*) and vector SK (lane SK) in vitro with the Single Tube Protein™ system using T3 RNA polymerase. The translation mixtures were analyzed by gel electrophoresis. The translation products of *MP13* cDNA was immunoprecipitation (*MP13*, + *FRA* Ab) with the FRA antibody, and with (lane +M peptide)/without M-peptide competition.

peptide)/without M-peptide competition. The results showed that *MP13* generated two major bands with apparent molecular weights of 62 and 32 kDa (Fig. 3). Both bands can be recognized by the *FRA* antibody and abolished by the competition with M-peptide, which showed that translated *MP13* products were especially recognized by *FRA* antibody. Next, we performed a non-denaturing gel mobility-shift assay to test whether the function of the *MP13* protein was related to GTP regulation. Translated *MP13* was bound to protein at room temperature for 20 min with/without the addition of GTP or GDT in binding mixture (20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 5 mM DTT, 50 μg/ml bovine serum albumin, 100 μg/ml sonicated salmon sperm DNA, and 10% glycerol). Protein complexes were separated on a 5% non-denaturing polyacrylamide gel. Gels were run at 150 V in 50 mM Tris/50 mM boric acid/1mM EDTA, dried and autoradiographed. The results revealed that the addition of GDP did not affect the binding of *MP13* protein to other proteins (Fig. 4, lanes GDP +). However, the addition of GTP aborted the binding of *MP13* protein to other proteins (Fig. 4, lanes GTP +).

Sequence comparison revealed that part of the *MP13* protein is more than 90% similar to the C-terminus of the activator of the small GTPase *Rab5*, *rabaptin-5* [12]. The C-terminus of *rabaptin-5* is predicted to be mainly α-helical and contain heptad repeats characteristic of coiled-coil domains. It might interact with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) that display coiled-coil domains [11] or it could be involved in the formation of complexes with other molecules acting upstream of SNAREs. Sequence comparisons of *MP13* protein with other members of the rab protein family did not show a high degree of similarity. Since there is no significant similarity within *rabaptin 5*, *rabaptin-3A* [11] and *rabin3* [2], it is hard to determine the actual function of *MP13*. However, our gel mobility-shift assay showed that the binding of *MP13* protein to other proteins was GTP related. Further investigation on the interaction of *MP13* protein with *Rab5* and *rabaptin-5* is in progress in order to reveal the function of *MP13*.

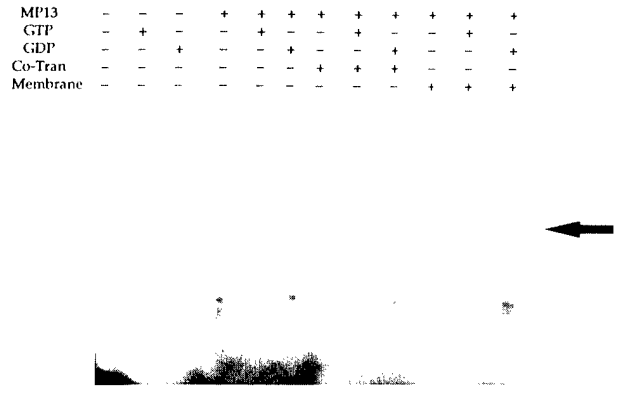

Fig. 4. The *MP13* binding activity to other proteins in the in vitro translation mixture. The *MP13* binding activity to other proteins was identified by gel mobility-shift assay on a 5% non-denaturing polyacrylamide gel. Gels were run at 150 V in 50 mM Tris/50 mM boric acid/1 mM EDTA, dried and autoradiographed. The addition of translated *MP13* products (*MP13*), 100 μM of guanosine 5′-*O*-(3-thiotriphosphate) for 20 min in the reaction mixture (GTP), and 100 μM of guanosine 5′-diphosphate for 20 min in the reaction mixture (GDP) were showed. Co-Tran represents the addition of reagents before the start of the translation of *MP13*; membrane represents the addition of 1 μl of microsomal membranes in the reaction mixture. '−' Indicates no addition of reagents and '+' is for addition of reagents. The arrow indicates the *MP13* band on the non-denaturing gel. Note that the addition of GTP aborted the *MP13* band in the gel.

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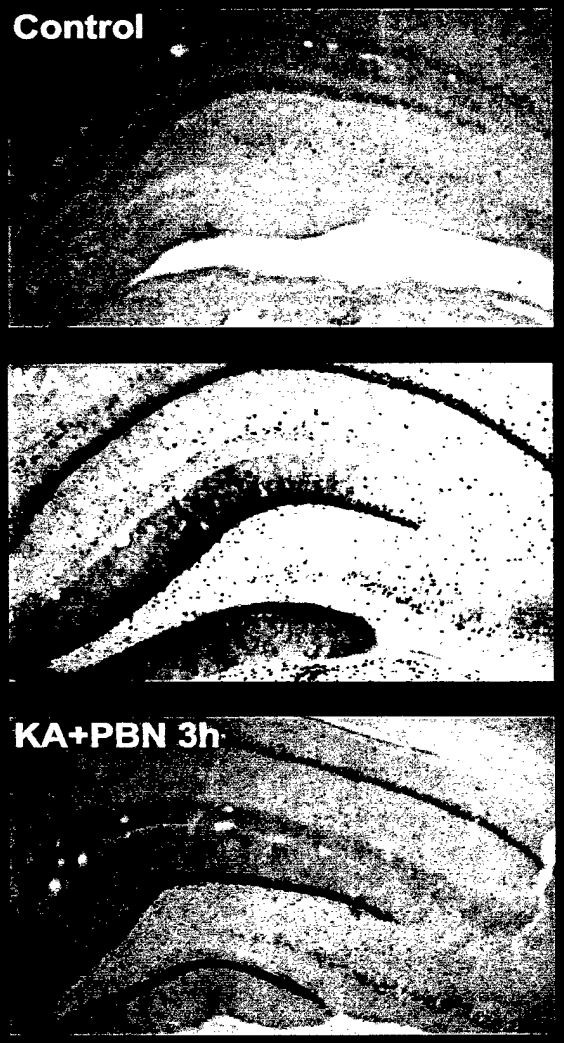
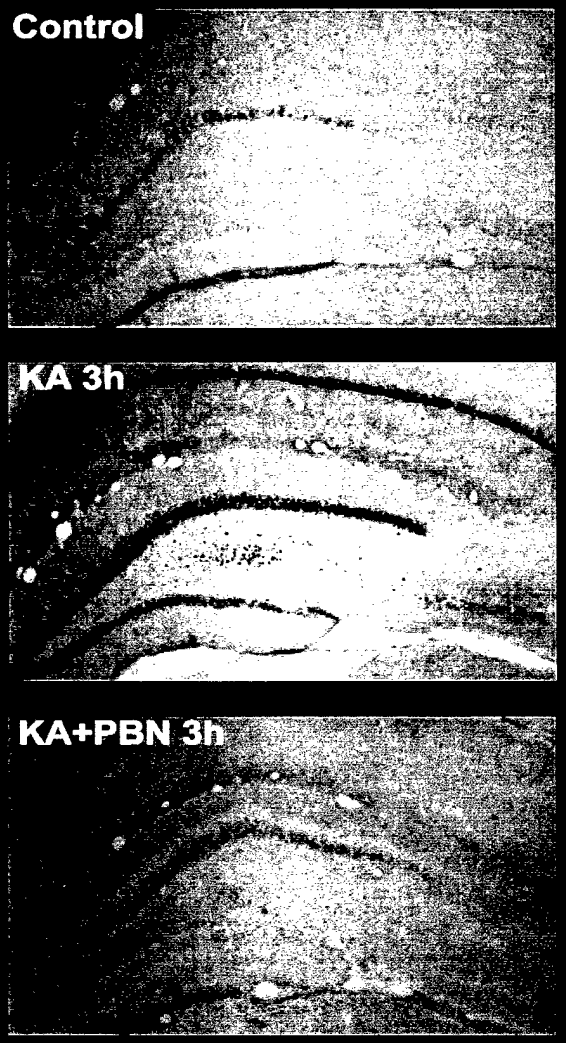
PBN Inhibits Kainic Acid-Induced AP-1 Activation

C-Fos



C-Jun



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1981-1982	Excellent in Teaching Award, Jilin Medical College
1982-1983	Fellowship for Chinese Graduate Student Study in USA, Chinese Government
1983-1984	Fellowship from Educational Commission for Foreign Medical Graduate, USA (ECFMG)
1984-1985	Teaching Assistantship, University of Rochester
1985-1986	Research Fellowship, Society of Physiology

PATENT AWARDED:

1. Methods of using alpha 2 agonist for the treatment of neurodegenerative diseases.
Inventor: **Guoying Bing,** and Eric Stone. 1993.
USA Patent Number: 5,252,816

2. A method for preventing and treating the degeneration of neurons.
Inventor, **Guoying Bing**, Nai-ying Zheng, Lei Jin, and Xin Lu, 1999
U.S. Serial No. **60,114,214**

PATENT PENDING:

1. A method for preventing and treating the neurodegenerative diseases.
Inventor, **Guoying Bing** and Jordan Tang, 2000

GENE BANK SUBMISSION:

1. Molecular cloning of a new gene for Fos-related antigen (FRA) in the kainic acid treated hippocampus.
Submitted by: **Guoying Bing,** Qiping Qi, Zhihuei Feng and Jau-Shyong Hong.
Accession Number: **U34932**

2. *Rat striatum genomic DNA of c-fos intron 3 and flanking cDNA sequence.*
Submitted by:, Zhihuei Feng, Kong L, Qiping Qi, No, S. Tiao, N., and **Guoying Bing**
Accession Number: **341647**

PROFESSIONAL POSITION:

2000-present **Associate Professor**
Department of Anatomy & Neurobiology, College of Medicine, University of Kentucky

1991-Present **Visiting Professor**
Beijing Institute for Neuroscience, Capital University of Medical Science, China

1997-2000 **Assistant Member**
Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation

1997-2000 **Adjunct Professor**
Department of Cell biology, University of Oklahoma, Health Science Center.

1993-1997 **Senior Fellow**
National Institute of Environmental Health Science

1991-1993 **Assistant Professor**
NYU Medical Center, Department of Psychiatry

1989-1991 **Staff Fellow**
NYU Medical Center, Department of Psychiatry

1983-1988 **Teaching Assistant and Tutor**

University of Rochester, School of Medicine, Department of Neurobiology & Anatomy

1978-1983 **Instructor**

Department of Anatomy, Jilin Medical College

RESEARCH INTERESTS:

Major research interests focus on the molecular and cellular mechanisms underlying the neurodegenerative diseases. Currently, there are three research projects are actively carried on in the laboratory: 1) the role of neuro-inflammatory processes in the etiology and pathophysiology of Parkinson's disease—A new animal model for Parkinson's disease. 2). Long-term neuronal adaptation to excitatory neurotoxicity---Molecular cloning long-term, differential expressed genes in the hippocampus after KA-induced epileptic seizures. and 3). The role of xenobiotic metabolite enzymes in the central nervous system---The effects of environmental and endogenous toxins on neurodegeneration.

RESEARCH GRANTS:

Active Support:

Principal Investigator for US Army Medical Research Grant-

Project Title: Protective Mechanisms of Nitrone Antioxidants in Kainic Acid Induced Neurodegeneration

Effective: June 1, 1999 through May 31, 2002

Total Amount: $540,000

Principal Investigator for NIH R01 Grant

Project Title: Microglia Activation Induces Parkinsonism in rats

Effective: December 1, 1999 through November 31, 2003

Total Amount: $830,000

Pending:

Principal Investigator for NIH R01 Grant

Project Title: Long-term Expression of Enkephalin in the hippocampus

Effective: June 1, 2000 through May 31, 2005

Total Amount: $1,150,000

Principal Investigator for Kentucky Spinal Cord and Head Injury Research Board

Project Title: Role of PBN and related nitrone antioxidants in spinal cord injury

Effective: January 1, 2001 through December 31, 2003

Total Amount: $299,831

TEACHING ACTIVITY:

Gross Human Anatomy 1979-1982, Jilin Medical College
Neuroanatomy 1988-1989, University of Rochester
Neuroscience 1998-1999, Oklahoma University

STUDENTS /POSTDOCTORAL/TRAINEES:

UNDERGRADUATE/GRADUATE RESEARCH ADVISOR

Riana Oomen, Fleming Scholar Summer Student 1997
Rebecca Summer, Fleming Scholar Summer Student 1998
Raha Neal, MD., Ph.D. Student 1999-
Xuan Nguyen, M.D., Ph.D. Student 2000-

POSTDOCTORAL FELLOWS

	CURRENT POSITION
Yi Zhang, M.D. 1991-1993	Editorial assistant, Society of Physiology
Lei Jin, MD. Ph.D. 1995-1999	Professor, Peking Union Medical University
Lingling Zhao, M.D., 1999-	Professor, Hunan Medical University
Toyoko Arimoto, Ph.D., 1999-	
Anyang Sun, Ph. D., 1999-	

PROFESSIONAL TRAINEES

Hyoung-Chun Kim, Ph.D., 1994-1996	Professor, Kangwon National University, Korea
Qiping Qi, Ph.D., 1995-1996	Director, Institute of National Academy of Preventive Medicine, China
Xianxi Liu, M.D., 1997	Professor, Shandong Medical University
Yahui Qi, M.D., 1998	Associate Professor, Capital University of Medical Science, Beijing

REVIEWER ACTIVITY:

Brain Research
Neuroscience Protocols
Neuroscience

Neuroscience Letter

Neurodegeneration

J. Neuroscience

Free Radical Biology and Medicine

INVITED LECTURES:

1. "Cografts of Adrenal Medullary cells with Neurotrophic producing Cells" Veterans Administration Hospital , Bedford, MA 01730, 1987.

2. "Transplantation of Adrenal Medullary, Carotid Body Glomus Cells with C6 Glioma Cells into the rat brain" Department of Anatomy, Boston University School of Medicine Boston, MA 02118, 1987.

3. "Neurotransplantation: Present and Future" Capital Institute of Medicine, Beijing, China, 1988.

4. 4, "Animal models used in neurotransplantation" New York University, Medical Center, New York, NY 10016, 1991

5. "Locus coeruleus lesions potentiate neurotoxic effects of MPTP in dopaminergic neurons of the substantia nigra" NIEHS/NIH, Research Triangle Park, NC 27709, 1993.

6. "Long-term genomic effects of administration of kainic acid in the rat brain" Centaur Pharmaceutical Inc., Sunnyvale, CA 94086, 1995.

7. "The regulation of the opioid peptide by seizure activities ----Role of long-term AP-1 transcription factors". Oklahoma Medical Science Foundation, City, OK 73104, December, 1996.

8. "The regulation of the opioid peptide by seizure activities ----Role of long-term AP-1 transcription factors". University of Oklahoma, Oklahoma Center for Neuroscience, Oklahoma City, OK 73104, January, 1997.

9. Capital University of Medical Science, Beijing, China. March,1997.

10. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" Kangwon National University, Korea. April, 1997.

11. "Long-term gene induction in the hippocampus by excitatory amino acid----A PCR-selected subtractive cloning methods" Shanghai Medical University, Shanghai, China. September,1998.

12. "Current trends in research for neurodegenerative diseases" Shandong Medical University, Shandong, China. September,1998

13. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" National Institute of Radiation Research, Ciba, Japan. June, 1999.

14. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" Yamagata University, School of Medicine, Yamagata, Japan, June, 1999

15. "Recent development of Molecular biological techniques in Neuroscience Research". Capital University

of Medical Science, Beijing, China. March, July, 1999.

16. "Microglia mediated neuronal death----A new animal model for Parkinson's disease" University of Missouri-Kansas City, School of Pharmacy, Kansas City, MS, August, 1999.

PROFESSIONAL SOCIETIES:

Society for Neuroscience

The New York Academy of Science

The Oxygen Society

COMMITTEE SERVICE:

Research Advisory Committee, University of Kentucky

Fleming Scholar Select Committee, Oklahoma Medical Research Foundation (1997-2000)

Graduate Faculty Committee, University of Oklahoma Health Sciences Center(1998-2000)

MAJOR EXPERTISE & PROFESSIONAL ACTIVITIES:

Supervised three graduate student, four postdoctoral fellows, two research associates, and two technician since 1991 to conduct research involving following techniques:

Molecular Biology:
- Molecular cloning,
- cDNA library construction and library screening
- PCR Techniques
- Differential Display
- Subtractive Cloning
- Gel mobility shift assay
- Northern, Southern, and Western blot analysis
- TUNNEL Methods for in situ staining of apoptosis.

Neurobiology:
- Light and Electronic Microscope Techniques
- Major Histological and Pathological Staining
- Immunohistochemistry
- *In situ* Hybridization
- Animal Models (rat, mouse, and monkey) for Parkinson's disease
- Neurotransplantation using fetal brain tissue, neuroblastoma, glioma, and primary cultured cells
- Neurochemical Analysis by HPLC for Monoamine System.

Cell Biology:
- Tissue Culture;
- Primary Neuronal and Glial Cultures;
- *In situ* hybridization on the Brian Slides and Culture Dishes.

PUBLICATIONS:

1. Gash, D.M., Notter, M.F.D., **Bing, G.**, Kordower, J.F. **(1986)** Neural implants into primates: Studies employing differentiated neuroblastoma cells. *Cell and Tissue Transplantation into the Adult Brain* pp. 37.

2. Hansen, J.T., **Bing, G.**, Notter, M.F.D., Gash, D.M. **(1987)** Ultrastructure of striatal implants of adult adrenal chromaffin cells in unilateral 6-OHDA lesioned rats. *Anat. Rec.* 218:56A.

3. **Bing, G.**, Notter, M.F.D., Hansen, J.T., Gash, D.M. **(1988)** Comparison of adrenal medullary, carotid body and PC12 cell grafts in 6-OHDA lesioned rats. *Brain Res. Bull.* 20:399-406.

4. Hansen, J.T., **Bing**, G., Notter, M.F.D., Gash, D.M. **(1988)** Paraneuronal grafts in unilateral 6-OHDA lesioned rats: Morphological aspects of adrenal chromaffin and carotid body glomus cell implants. In: *Transplantation into Mammalian CNS* (D.M. Gash and J. R. Sladek, Jr., Editors) Elsevier, Amsterdam, *Prog Brain Res*, 78:535-542.

5. Gash, D.M., Notter, M.F.D., Hansen, J.T., **Bing, G.**, Okawara, S.H. **(1988)** Human organ donor adrenals: Fine structure, plasticity and viability. In: *Transplantation into Mammalian CNS* (D.M. Gash and J. R. Sladek, Jr., Editors) Elsevier, Amsterdam, *Prog Brain Res.* 78:559-565.

6. Kordower, J.H., **Bing, G.**, Fiandaca, M.S., Sladek Jr., J.R., Gash, D.M. **(1988)** Tyrosine hydroxylase-immunoreactivity somata within the primate subfornical organ: Species specificity. *Brain Res.* 461:221-229.

7. Hansen, J.T., **Bing, G.**, Notter, M.F.D., Gash, D.M. **(1989)** Adrenal chromaffin cells as transplants in animal models of Parkinson's disease. *J. Electron Microscopy Tech.* 12:308-315.

8. **Bing, G.**, Notter, M.F.D., Hansen, J.T., Kellogg, C., Gash, D.M. **(1990)** Cografts of adrenal medulla with C6 glioma cells in rats with 6-OHDA induced lesions. *Neurosci.* 34:687-697.

9. **Bing, G.**,. Neurotransplantation: The Present and Future. In: *Neurotransplantation* (S. Jiao and **G. Bing**, Editors) Science Press, Beijing, China.

10. **Bing, G.**, Filer, D., Miller, J.C., Stone, E.A. **(1991)** Noradrenergic activation of immediate early genes in rat cortex. *Molec. Brain Res.* 11:43-46.

11. Stone, E.A., Zhang, Y., John, S., **Bing, G. (1991)** C-fos response to administration of catecholamine into brain by microdialysis. *Neurosci. Lett.* 133:33-35.

12. **Bing, G.**, Chen, S., Zhang, Y., Hillman, D., Stone, E.A. **(1992)** Noradrenergic-induced expression of c-fos in rat cortex: neuronal localization. *Brain Res.* 140:260-264.

13. Stone, E.A., **Bing G.**, John S.M., Zhang, Y., Filer, D. **(1992)** Cellular localization of responses to catecholamine in brain tissue. *Prog. Brain Res.* 94:303-307.

14. Stone, E.A., John, S.M., **Bing, G.**, Zhang, Y. **(1992)** Studies on the cellular localization of biochemical responses to catecholamines in the brain. *Brain Res. Bull.* 29:285-288.

15. **Bing, G.**, Stone, E.A., Zhang, Y., Filer, D. **(1992)** Immunohistochemical studies of noradrenergic-induced expression of c-fos in the rat CNS. *Brain Res.* 592:57-62.

16. Stone, E.A., Zhang, Y., John, S., Filer, D., **Bing, G. (1993)** Effect of locus coeruleus lesion on c-fos expression in the cerebral cortex caused by yohimbine injection or stress. *Brain Res.* 19:181-185.

17. Stone, E.A., Manavalan, J.S., Basham, D.A., **Bing, G. (1994)** Effect of yohimbine on nerve growth factor mRNA and protein levels in rat hippocampus. *Neurosci. Lett.* 14:11-13.

18. **Bing, G.**, Zhang, Y., Watanabe, Y., McEwen, B.S., Stone, E.A. **(1994)** Locus coeruleus lesions potentiate neurotoxic effects of MPTP in dopaminergic neurons of the substantia nigra. *Brain Res.* 668:261-265.

19. Hiller, J., Zhang, Y., **Bing, G.**, Gioannini, T., Stone E., Simon, E. **(1994)** Immunohistochemical Localization of mu-opioid receptors in rat brain using antibodies generated against a peptide sequence present in a purified mu-opioid binding protein. *Neurosci.* 62:829-841.

20. McMillian, M., Kong, L.-Y., Sawin, S.M., Wilson, B., Das, K., Hudson, P., Hong, J.-S., **Bing, G. (1995)** Selective killing of cholinergic neurons by microglial activation in basal forebrain mixed neuronal/glial cultures. *Biochem. Biophys. Res. Commun.* 215:572-577.

21. Das, K.P., McMillian, M., **Bing, G.**, Hong, J.-S. **(1995)** Modulatory effects of [Met^5]- enkephalin on interleukin-1b secretion from microglia in mixed brain cell cultures. *J. Neuroimmuno.* 62:9-17.

22. Perez-Otano, I., McMillian, M., **Bing, G.**, Hong, J.-S., Pennypacker, K. **(1996)** Induction of NF-kB-like transcription factors in brain areas susceptible to kainate toxicity. *Glia.* 16:306-315.

23. **Bing, G.**, Wilson, B., McMillian, M., Feng, Z., Qi, Q., Kim, H., Wang, W., Jensen, K., Hong, J.-S. **(1996)** Long-term expression of Proenkephalin and prodynorphin in the rat brain after systemic administration of kainic acid ——an *in situ* hybridization study. in *Neurodegenerative Disease,* ed. by G. Flskum, Plenum Press, pp 8-18.

24. **Bing, G.**, McMillian, M.,Kim, H., Pennypacker, K., Feng, Z., Qi, Q., Kong, L.-Y, Iadarola, M., Hong, J.-S. **(1996)** Long-term expression of the 35-kDa fos-related antigen (FRA) in rat brain after kainic acid treatment. *Neurosci.* 73:1159-1174.

25. Kim, H., Pennypacker, K., **Bing, G.**, Bronstein, D., McMillian, M., Hong, J.-S. **(1996)** the effects of dextromethorphan on kainic acid-induced seizures in the rat. *J. Neurotoxic.* 17:375-386.

26. Kong, L.-Y., McMillian, M., **Bing, G.**, Hudson, P.M., Hong, J.-S. **(1996)** The effects of the HIV-1 envelope protein gp 120 on the production of nitric oxide and proinflammatory cytokines in mixed glial cell cultures. *Cell Immunol.* 172:77-83.

27. **Bing, G.**, Wang, W., Qi, Q., Feng, Z., Jin, L., Bing, R., Hong, J.-S. **(1997)** Long-term expression of Fos-related antigen and transient expression of FosB associated with seizures in the hippocampus and striatum. *J. Neurochem.* 68:272-279.

28. Kim, H., **Bing, G.**, Hong, J.-S. **(1997)** Dextromethorphan blocks opioid peptide gene expression in the rat hippocampus induced by kainic acid. *Neuropeptides.* 31:05-112.

29. **Bing, G.**, Wilson, B., Hudson, P., Jin, L., Feng, Z., Zhang, W., Bing, R. **(1997)** A single dose of kainic acid elevates the levels of enkephalins and activator protein-1 transcription factors in the hippocampus for up to 1 year. *Proc. Natl. Acad. Sci., USA.* 94:9422-9427.

30. Simpson, J.N., Zhang, W.Q., **Bing, G.**, Hong, J.-S. **(1997)** Kainic acid-induced sprouting of dynorphin- and enkephalin-containing mossy fibers in the dentate gyrus of the rat hippocampus. *Brain Res.* 747:318-323

31. Feng, Z., Zhang, W., **Bing, G.**, Hundson, P., Feng, W., Hong, J.-S. **(1997)** Characterization of the long-lasting activator protein-1 complex induced by kainic acid treatment. *Brain Res.*770:53-59.

32. Chen, S., Ren, Y.Q., **Bing, G.**, Hillman, D.E. **(1998)** Transient c-*fos* gene expression in cerebellar development and functional stimulation. *Brain Res* 795:87-97.

33. Gupta, R.P., **Bing, G.**, Hong, J.S., Abou-Donia, M.B. **(1998)** cDNA cloning and sequencing of Ca^{2+}/calmodulin-dependent protein kinase II subunit and its mRNA expression in diisopropyl phosphorofluoridate (DFP)-treated hen central nervous system. *Mol Cell Biochem* 181:29-39.

34. Kim H.C., **Bing, G,** Jhoo, W.K., Ko, K.H., Kim, W.K., Lee, D.C., Shin, E.J., Hong, J.S. **(1999)** Dextromethorphan modulates the AP-1 DNA-binding activity induced by kainic acid. *Brain Res* 824:125-132.

35. Hensley, K., Floyd, R.A., Zheng, N.Y., Nael, R., Robinson, K.A., Nguyen, X., Pye, Q.N., Stewart, C.A., Geddes, J., Markesbery, W.R., Patel, E., Johnson, G.V.M., **Bing, G. (1999)** p38 Kinase is activated in the Alzheimer's disease brain. *J. Neurochem.* 72:2053-2058.

36. Feng, Z., Chang, R.C., **Bing, G.,** Hudson, P., Tiao, N., Jin, L., Hong, J.S. **(1999)** Long-term increase of Sp-1 transcription factors in the rat hippocampus after kainic acid treatment. *Brain Res* 69:144-148.

37. Floyd, R.A., Robinson, K.A., Stewart, C.A., **Bing, G.,** Hensley, K. **(1999)** Neuroinflammatory events and signal transduction processes are involved in neurodegeneration. In: Free Radicals in Brain Pathophysiology. (Cadenas, E., Packer, L, Poli, G., Ed.) pp. 109-126, Marcel Decker, NY.

38. Kim, H.C., Jhoo, W.K., Choi, D.Y., Im, D.H., Shin, E.J., Suh, J.H., Floyd, R.A., **Bing, G. (1999)** Protection of methamphetamine nigrostriatal toxicity by dietary selenium. *Brain Res.* 851:76-86.

39. Floyd, R.A., Robinson, K.A., Stewart, C.A., **Bing, G.,** Hensley, K. **(1999)** Neuroinflammatory events and signal transduction processes are involved in neurodegeneration. In: Free Radicals in Brain Pathophysiology. (Cadenas, E., Packer, L, Poli, G., Ed.) pp. 109-126, Marcel Decker, NY.

40. Kim H.C., **Bing, G,** Jhoo, W.K., Ko, K.H., Kim, W.K., Lee, D.C., Shin, E.J., Hong, J.S. **(1999)** Dextromethorphan modulates the AP-1 DNA-binding activity induced by kainic acid. *Brain Res* 824:125-132.

41. Hensley, K., Floyd, R.A., Zheng, N.Y., Nael, R., Robinson, K.A., Nguyen, X., Pye, Q.N., Stewart, C.A., Geddes, J., Markesbery, W.R., Patel, E., Johnson, G.V.M., **Bing, G. (1999)** p38 Kinase is activated in the Alzheimer's disease brain. *J. Neurochem.* 72:2053-2058.

42. Feng, Z., Chang, R.C., **Bing, G.,** Hudson, P., Tiao, N., Jin, L., Hong, J.S. **(1999)** Long-term increase of Sp-1 transcription factors in the hippocampus after kainic acid treatment. *Brain Res* 69:144-148.

43. Kim, H.C., **Bing, G.,** Jhoo, W.K., Ko, K.H., Kim, W.K., Suh, J.H., Kim, S.J., Kato, K., Hong, J.S. **(2000)** Changes of hippocampal Cu/Zn-superoxide dismutase after kainate treatment in the rat. *Brain Res.* 853:215-226.

44. Kim, H.C., Jhoo, W.K., Ko, K.H., Kim, W.K., **Bing, G.,** Kwon, M.S., Shin, E.J., Huh, J.H., Lee, Y.G., Lee, D.W. **(2000)** Prolonged exposure to cigarette smoke blocks the neurotoxicity induced by kainic acid in rats. *Life Sci.* 66:317-326.

45. Kim, H.C., Jhoo, WK, **Bing G**, Shin, E.J., Wie, M.B., Kim, W.K., and Ko, K.H. **(2000)** Phenidone prevents kainate-induced neurotoxicity via antioxidant mechanisms. *Brain Res.* 874:15-23.

46. Kim, H.C., Jhoo, W.K., Shin, E.J., **Bing, G. (2000)** Selenium deficiency potentiates methamphetamine-induced nigral neuronal loss; comparison with MPTP model. *Brain Res.* 862:247-252

47. Lu, X**., Bing, G**, and Hagg, T. **(2000)** Naloxone prevents microglia-induced degeneration of dopaminergic substantia nigra neurons in adult rats, *Neuroscience*, 97:285-291

48. Feng, Z., Qi, Q., Wilson, B., McMillan, M., Kim K.H., Hong, J. **Bing, G. (2000)** Cloning and expression of MP 13, an antigen immunoreactive with antibody against FOS-related antigen, from rat hippocampus after systemic kainic acid treatment. . *Neurosci Lett.* 296:129-132.

49. Floyd, R.A., Hensley, K., **Bing, G. (2000)** Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics. *J. Neural Transm.* 60:387-414..

50. **Bing, G.,** Lu, X., Zheng, N., Jin, L., Kim, H. **(2000)** Microglia activation induced dopaminergic cell death in the substantia nigra. Submitted to *Nature Neurosci.*

51. **Bing, G.**, Neal, R., Zheng, N., Jin, L., Zhu, M., Kim, H. **(2000)** Induction of epoxide hydrolase in neurodegenerative diseases. Submitted to *J. Neurosci.*

RELEVANT ABSTRACTS:

1. **Bing, G.**, Notter, M.F.D., Kellogg, C., Gash, D.M. **(1986)** Implants of PC 12 cells into rats with unilateral nigrostriatal lesions. *Neurosci Abstr.* 12:1288.

2. **Bing, G.**, Notter, M.F.D., Hansen, J.T., Kellogg, C., Kordower, J.H., Gash, D.M. **(1987)** Adrenal Medullary transplants IV. Cografts with growth factor producing cells. *Neurosci Abstr.* 13:16.

3. Lory, J., Kordower, J.H., **Bing, G.**, Sladek, J.R., Jr., Gash, D.M. **(1987)** Species specific tyrosine hydroxylase immunoreactive cell group in the subfornical organ: Presence in the monkey but not rat brain. *Neurosci Abstr.* 13:1336.

4. **Bing, G.**, Jiao, S., Notter, M.F.D., Hansen, J.T., Gash, D.M. **(1988)** Cografts of adrenal medulla with peripheral nerve in the dopamine denervated rat striatum. *Neurosci. Abstr.* 14:735.

5. **Bing, G.**, Vielkind, U., Bohn, M.C. **(1989)** Glucocorticoid receptor expression in primary hippocampal neuronal cultures. *Neurosci. Abstr.* 15:717.

6. **Bing, G.**, Stone, E.A., Miller, J.C., Friedhoff, A.J., Filer, D. **(1990)** Beta adrenoceptor-induced expression of early response genes in the rat cerebral cortex. *Neurosci. Abstr.* 16:2.

7. Watanabe, Y., Angulo, J., **Bing, G.**, Stone, E.A., McEwen, B.S. **(1991)** Effects of repeated restraint stress in rats on neuroendocrine and molecular markers of the brains response. *Neurosci. Abstr.* 17:83.

8. **Bing, G.**, Zhang, Y., Filer, D., Stone, E.A. **(1991)** Immunohistochemical identification of c-fos protein in rat brain after noradrenergic stimulation. *Neurosci. Abstr.* 17:1357.

9. Zhang, Y., Bing, **G.**, Filer, D., Stone, E.A. **(1992)** Effect of locus coeruleus (LC) lesion on c-Fos response to metrazol (PTZ) in rat brain. *Neurosci. Abstr.* 18:1375.

10. **Bing, G.**, Zhang, Y., Stone, E.A. **(1992)** Protective action of locus coeruleus noradrenergic system on substantia nigra (SN) of mice treated with MPTP. *Neurosci. Abstr.* 18:1375.

11. **Bing, G.**, Manavalan, J.S., Stone, E.A. **(1993)** Hippocampal NGF increases following yohimbine injection. *Neurosci. Abstr.* 19:50.

12. Stone, E.A., Zhang, Y., **Bing, G. (1993)** The role of the noradrenergic system in central c-fos responses. *Neurosci. Abstr.* 19:17.

13. Hiller, J.M., **Bing, G.**, Stone, E., Gioannini, T.I., Simon, E.J. **(1993)** Immunohistochemical localization of μ opioid receptors in rat brain with antibodies against a peptide sequence derived from a purified opioid receptor binding protein. *Neurosci. Abstr.* 19:116.

14. Perez-Otano, I., Pennypacker, M.K., McMillian, M.K., **Bing, G.**, Hong, J.S. **(1994)** NF-kB transcription factors are increased in brain areas susceptible to kainate toxicity. Neurosci. *Abstr.* 20:50.

15. Shao, Y., **Bing, G.**, Chen, K.H., Kufuor, N.K., Qi, Q., Mccarthy, K.D., Hu, P.C., Hong, J. **(1994)** Adenovirus and adeno-associate virus mediated gene transfer in rat brain cells. *Neurosci. Abstr.* 20:94.

16. Kim, H., Pennypacker, M.K., **Bing, G.**, J.S. Hong. **(1994)** Effects of dextromethorphan on kainate acid-induced seizure in rat. *Neurosci. Abstr.* 20:244.

17. **Bing, G.**, Wu, G., Kim, H., McMillian, M., Qi, Q., He, X., J.S. Hong. **(1994)** D1 dopaminergic agonists increase the expression of dynorphin and c-fos in primary striatal culture of rat. *Neurosci. Abstr.* 20:257.

18. **Bing, G.**, McMillian, M., Kim, H., Pennypacker, K., Feng, Z., Qi, Q., Kong, L., Chan, J., Wilson, B., Hong, J.S. **(1995)** Long-term expression of the 35-kDa fos-related antigen (FRA) in rat brain after kainic acid treatment. *Neurosci. Abstr.* 21:127.1.

19. Kong, L.-Y., McMillian, M., **Bing, G.**, Hudson, P.M., Hong, J.S. **(1995)** The effects of the HIV-1 envelope protein gp 120 on the production of nitric oxide and proinflammatory cytokines in unprimed or interferon g- primed glial cell cultures. *Neurosci. Abstr.* 21:352.18.
20. Feng, Z., **Bing, G.**, Qi, Q., Wilson, B., McMillian, M., Pennypacker, K., Iadarola, M., Hong, J.S. **(1995)** Cloning and analysis of fos-related antigen from rat hippocampus after systemic of kainic acid. *Neurosci. Abstr.* 21:521.14.

21. McMillian, M., Kong, L.-Y., Sawin, S.M., Wilson, B., Das, K., Hudson, P., Hong, J.S., **Bing, G. (1995)** Selective killing of cholinergic neurons by microglial activation cell cultures. *Neurosci. Abstr.* 21:679.18.

22. Kim, H.C., Kim, S.J., **Bing, G.**, Hong, J.S. **(1995)** The role of hippocampal Cu, Zn-superoxide dismutase (SOD-1) in kainic acid-induced neuronal degeneration. *Neurosci. Abstr.* 21:836.21.

23. Simpson, J., **Bing, G.**, Feng, Z., Wilson, B., Hong, J.S. **(1996)** Long-term effects of kainic acid-induced seizures on the opioid peptides and AP-1 transcription factors I. Morphological studies. *Neurosci. Abstr.* 22:519.7.

24. Feng, Z., Zhang, W., **Bing, G.**, Simpson, J., Hudson, P., Hong, J.S. **(1996)** Long-term effects of kainic acid-induced seizures on the opioid peptides and AP-1 transcription factors II. Biochemical studies. *Neurosci. Abstr.* 22:519.8.

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